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PRODUCTION OF CLAVIFORMIN BY SOIL PENICILLIA[1]

BY A. G. LOCHHEAD[2], F. E. CHASE[3], AND G. B. LANDERKIN[3]

Abstract

Of various strains of fungi isolated from soil that showed antibiotic activity against Gram-positive and Gram-negative bacteria, two species of *Penicillium*, producing culture filtrates of high antibacterial titre, were studied in more detail. The active substance, when crystallized, was considered to be claviformin (clavacin, clavatin, patulin). Studies on the production of the antibiotic, its assay, and its extraction from culture filtrates are described.

Introduction

In 1942 Wiesner (18) reported the concentration of a substance from culture filtrates of *Aspergillus clavatus* that inhibited growth of *Staphylococcus aureus*. Waksman, Horning, and Spencer (17) likewise found the same fungus to yield an antibiotic substance, obtained as a crude concentrate, that was active against a variety of Gram-negative, as well as Gram-positive, bacteria and that was named clavacin. At the same time Chain, Florey, and Jennings (4) isolated from culture filtrates of *Penicillium claviforme* a crystalline compound, active against Gram-negative and Gram-positive organisms, to which the name claviformin was given. In 1943 Anslow, Raistrick, and Smith (1) and Raistrick *et al.* (13) reported the isolation of patulin, an antibiotic substance produced by *Penicillium patulum* and *P. expansum*, obtainable in crystalline form, with the empirical formula $C_7H_6O_4$, and for which the structure anhydro-3-hydroxymethylene-tetrahydro-μ-pyrone-2-carboxylic acid was proposed.

From the metabolism solution of Wiesner's strain of *A. clavatus*, Bergel *et al.* (2) isolated, as a crystalline entity, an active substance to which the name clavatin was applied and that was found identical with claviformin. Furthermore, Bergel *et al.* (3) established the identity of clavatin with patulin, which latter was shown by Chain *et al.* (5) to be similar to claviformin. In the meantime Hooper *et al.* (9) reported clavacin to be identical with patulin, a finding confirmed by Katzman *et al.* (11). It appears, therefore, that claviformin, clavacin, clavatin, and patulin are the same substance. Since claviformin appears to have been the term first used for the crystalline material this name is used in this report.

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In addition to the fungi mentioned, *Aspergillus giganteus* was found by Florey, Jennings, and Philpot (7) to produce claviformin. Karow and Foster (10) also found the same active material in culture filtrates of species of *Gymnoascus* and *Penicillium*, while Kent and Heatley (12) reported its production by *Penicillium urticae* and *Aspergillus terreus*.

It is generally agreed that claviformin is toxic at concentrations that make therapeutic use unlikely for injection into animals. Less is known as regards its value for external use. That it may have possible value as an antifungal agent is suggested by the recent findings of Herrick (8), particularly since most fungal diseases of man and animals are local and superficial. Its action against other fungi *in vitro*, including plant pathogens (1, 15, 16), suggest possibilities for further application. The present report deals with studies on the production, assay, and extraction of claviformin formed by penicillia isolated from soil, which were found to be active producers of this antibiotic.

Experimental

Though several species of fungi isolated from soil were found to yield a substance regarded as claviformin, studies were confined to two species of *Penicillium* (No. 4 and No. 08) isolated from samples of garden soil stored at 25° C., which had received, respectively, weekly additions of suspensions of *Salmonella typhi-murium* for three months, and *Pseudomonas aeruginosa* for 10 months. Isolations were made by plating on glucose-nitrate agar. Preliminary tests indicated pronounced antibacterial effects against a variety of Gram-negative bacteria as well as *Staphylococcus aureus*. Culture filtrates of the fungi grown in modified Czapek-Dox medium (6) showed a high antibacterial titre, inhibiting *S. aureus* usually at 1-320, and *Escherichia coli* at 1-640 dilution. Since No. 4 occasionally gave titres against *E. coli* of 1-1000, chief attention was given to this mould.

Method of Assay

To measure the antibacterial potency of antibiotic solutions a more accurate procedure is required than that provided by the serial dilution method, which is not only laborious, but gives at best an approximation. The procedure adopted was the cylinder-plate method of Schmidt and Moyer (14) for penicillin assay, using *E. coli* as test organism in place of *S. aureus*. Both organisms were used in the earlier experiments, but, since results were analogous, assays were continued with *E. coli* alone.

Since the diameter of the zone of inhibition by the cylinder-plate method is not directly proportioned to the antibiotic potency of the test solution, for purposes of comparison a system of evaluation based on arbitrary units was used. Measurement of the zone of inhibition produced by definite dilutions of a wide series of culture filtrates made it possible to establish a standard curve based on assigning an arbitrary value of 10 units to an inhibition zone of 25 mm. diameter (Fig. 1). By using appropriate dilutions, satisfactory evaluation could be made of unknown solutions.

The relation between the inhibition zone by the cylinder-plate method and the bacteriostatic potency as determined by the serial dilution method varies with the antibiotic and with the test organism. Though inhibition zones

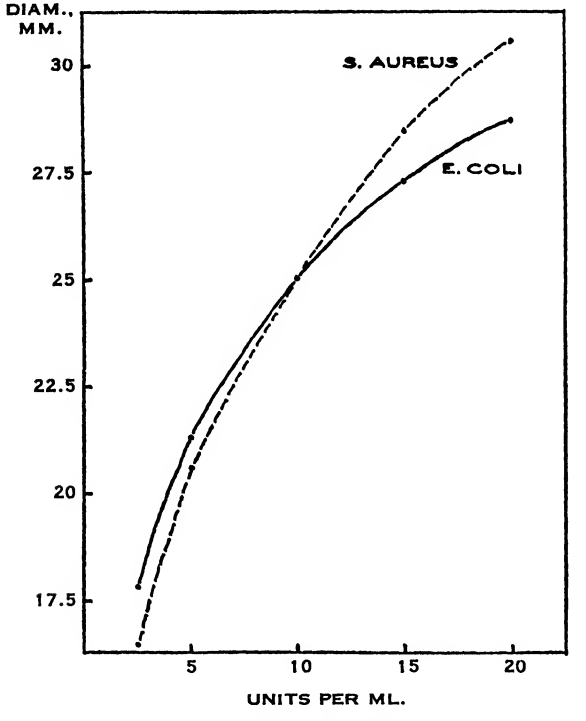

FIG. 1. *Standard curves for S. aureus and E. coli for assay of potency by cylinder-plate method.*

against *S. aureus* and *E. coli* are of approximately equal diameter, serial dilution tests, whether on the culture filtrate or the crystalline material, showed that the active principle possesses considerably greater antibacterial potency against *E. coli* than against *S. aureus* (cf. Table IV).

Production of Antibiotic Substance

The solution for production of the antibiotic, used as control medium in the experiments, was the modified Czapek-Dox solution described by Clutterbuck, Lovell, and Raistrick (6), consisting of glucose, 40 gm.; sodium nitrate, 3 gm; potassium dihydrogen phosphate, 1 gm.; potassium chloride, 0.5 gm.; magnesium sulphate heptahydrate, 0.5 gm.; ferrous sulphate heptahydrate, 0.01 gm.; distilled water, 1000 ml. The medium was dispensed in 80 ml. quantities in 300 ml. Erlenmeyer flasks, sterilized (pH = 4.0), inoculated (1 ml. per flask) with a spore suspension of the mould prepared from cultures varying from 5 to 20 days old on Czapek-Dox agar, and incubated (unless otherwise specified) at 25° C. For the assay the contents of duplicate flasks

were mixed, filtered, and appropriate dilutions used for the cylinder-plate tests.

Effect of Temperature

The effect of temperature of incubation on production of claviformin by *Penicillium* sp. (No. 4) is shown in Fig. 2. Though relatively little difference was noted between 20° and 25° C., production of the antibiotic was much decreased at 30° C. No growth of the mould occurred at 37° C.

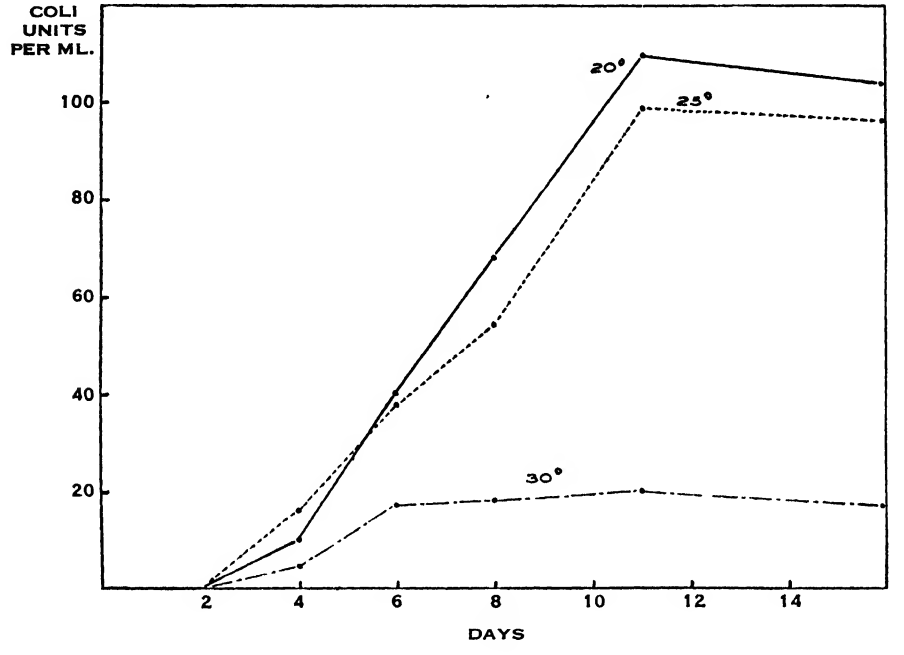

FIG. 2. *Effect of temperature on production of claviformin.*

Carbohydrate Source

The results of comparative tests with glucose, sucrose, lactose, starch, and dextrin as source of carbohydrate are shown in Fig. 3. Glucose proved the most suitable carbohydrate of those used for antibiotic production, giving a maximum of over 100 coli units per ml., corresponding to a titre of approximately 1-800 by the serial dilution method. With lactose, production failed to reach its peak during the course of the experiment.

Effect of Addition of Extracts

The production of certain antibiotics (e.g. penicillin) is known to be stimulated by the addition of corn steep liquor to the culture medium. Katzman *et al.* (11) found that the yield of claviformin (clavacin) by *Aspergillus clavatus* was improved by adding corn steep liquor or yeast extract. The effect of various extracts on claviformin formation by *Penicillium* sp. (No. 4) was studied in a test in which 1% yeast extract, 8% corn steep liquor, and 20%

soil extract were incorporated respectively in the Czapek-Dox solution. Soil extract was prepared by autoclaving 1 kgm. soil with 1 litre of water and filtering.

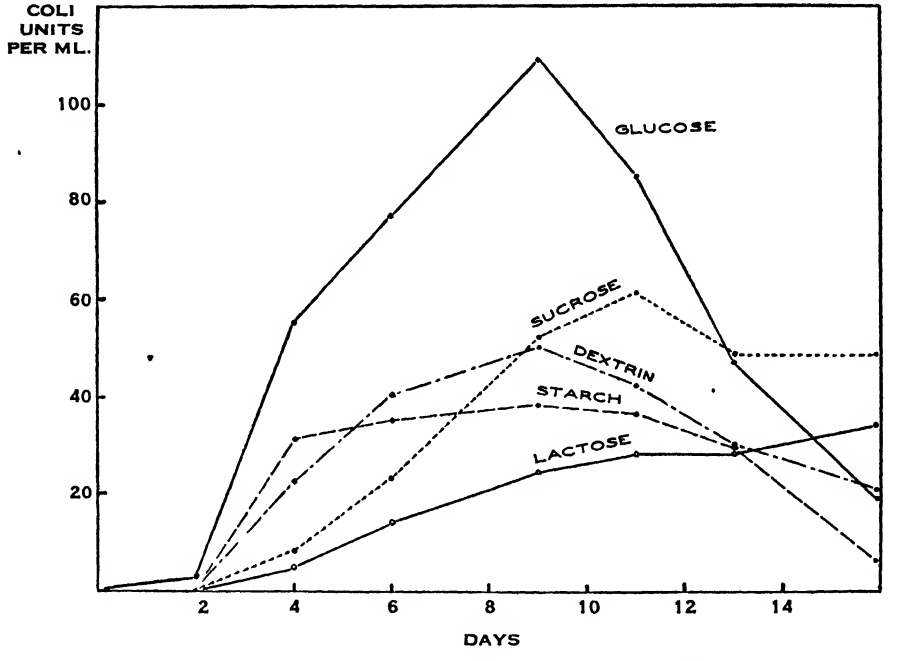

FIG. 3. *Effect of carbohydrate source on production of clavıformin.*

The results, shown in Fig. 4, indicate that claviformin formation was depressed by both yeast extract and corn steep liquor. With soil extract no such effect was noted, a higher maximum being reached than in the control solution. During the period of active claviformin production the solution remains acid (below pH = 5). The rise in pH value above 5 as the culture ages is associated with a decline in the antibacterial potency.

Effect of Heavy Metals

The effect of replacing the iron in the Czapek-Dox solution, in whole or in part, by other heavy metals was studied in an experiment in which manganese, zinc, and copper, used both singly and in combination with iron, were compared with iron alone. The metals were all employed as sulphates (iron as ferrous sulphate), in concentration of 0.001% when used alone or 0.0005% in combination to maintain the total concentration of metallic salts at approximately the same level. As noted in Table I, maximum claviformin production was attained with iron alone, manganese being somewhat less favourable. The different effects of the various metals on the metabolism of the mould are reflected also in the pH values. Though zinc, alone or with iron, favours more rapid formation of the antibiotic than copper, the increased pH of the solution causes a more rapid decline in potency than in the presence of the

latter element, the inclusion of which maintains a continued acid reaction accompanied by more gradual production of the antibiotic.

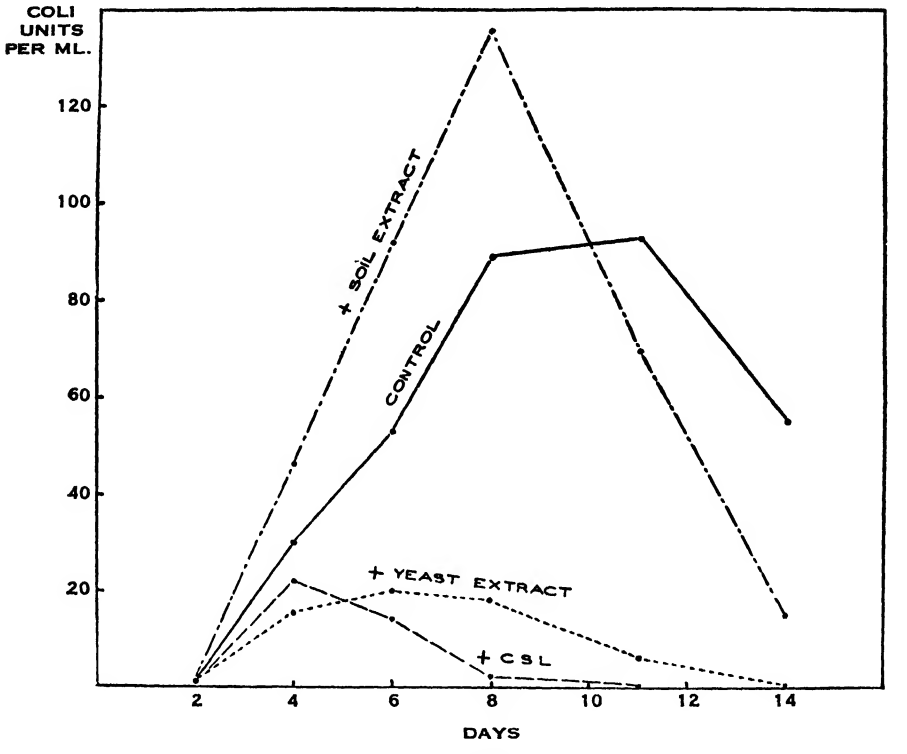

FIG. 4. *Effect of various extracts on production of claviformin.*

TABLE I

EFFECT OF REPLACING IRON, IN WHOLE OR IN PART, BY OTHER HEAVY METALS ON PRODUCTION OF CLAVIFORMIN BY *Penicillium* SP. (NO. 4)

Metal, as sulphate*	Coli units per ml. and pH									
	Incubation, days									
	3		5		8		10		13	
	Units	pH	Units	pH	Units	pH	Units	pH	Units	pH
Fe ($FeSO_4$, as in Czapek-Dox; control)	15	4 8	51	4 2	100	4 2	76	4 8	50	6 1
Mn	16	4 2	59	3 7	81	3 7	71	3 9	62	3 9
Zn	7	4 5	20	4 2	42	4 4	42	5 1	7	7 2
Cu	0	4 4	2	3 7	17	3 6	12	3 4	15	3 2
Fe + Mn	12	4.5	37	4 1	76	3 9	66	4 1	58	4 0
Fe + Zn	9	5 4	42	4 9	36	5 4	18	6 8	5	7.5
Fe + Cu	1	4 6	21	3 8	39	3 5	43	3 1	49	3.1

* *Concentration: used singly, 0.001%; in combination, 0.0005%.*

Stationary vs. Shaken Cultures

Antibiotic formation in stationary (surface) and shaken (submerged) cultures was compared in a series of tests using three media. The results (Table II) show that for the mould in question, claviformin production was greater in surface culture, though under submerged conditions excellent growth was obtained.

TABLE II

PRODUCTION OF CLAVIFORMIN BY *Penicillium* SP. (NO. 4) IN STATIONARY (SURFACE) AND SHAKEN (SUBMERGED) CULTURES

Medium	Cultures	Coli units per ml.				
		Incubation, days				
		2	4	6	8	10
Czapek-Dox	Stationary	2	35	46	66	42
	Shaken	0	5	6	10	6
Czapek-Dox + soil extract	Stationary	3	36	60	70	42
	Shaken	2	2	13	26	25
Raulin-Thom	Stationary	1	21	40	66	39
	Shaken	0	0	3	12	7

Extraction of Active Substance

Larger scale experiments to note the suitability of various solvents for recovery of the active substance were carried out in 2000 ml. Erlenmeyer flasks containing 500 ml. culture fluid, inoculated with 10 ml. spore suspension. Active cultures (pH not over 5.0) were filtered through paper, the contents of four flasks being usually combined for each trial. The results of 25 experiments, including treatments with different solvents are summarized in Table III.

TABLE III

EFFECT OF VARIOUS PROCEDURES ON EXTRACTION OF CLAVIFORMIN FROM CRUDE CULTURE FILTRATES (*Penicillium* SP. NO. 4)

Treatment of culture filtrate	No. of trials	Recovery of active substance (average), %	Coli units per mgm. crude residue (average)
Direct extraction—ether	1	6	—
Direct extraction—chloroform	1	2	—
Concentration—ethyl acetate extraction	2	84	20.9
Darco adsorption—acetone elution	2	49	8.7
Norit adsorption—acetone elution	7	44	5.5
Norit adsorption—ether elution	2	14	—
Norit adsorption—ethanol elution	2	21	5 0
Norit adsorption—chloroform elution	3	18	28.4
Norit adsorption—ethyl acetate elution	5	75	25.6

The most convenient treatment included adsorption of the active material on charcoal (1.5%) followed by elution, generally with a total of 200 to 300 ml. of solvent per litre of original culture, added in three or four successive treatments. Of the various solvents used ethyl acetate proved the most effective, permitting an average recovery of 75% of the active principle (maximum 91%). Acetone was less effective while much smaller recovery was obtained with ether, ethanol, or chloroform. In all cases evaporation of the eluate left a brownish, somewhat gummy residue, which was darker in proportion to the amount of inert material extracted. As noted in Table III, the crude residues from ethyl acetate and chloroform contained much higher concentrations of claviformin than those from other solvents, assaying approximately 50 to 60% pure substance. (Crystalline claviformin = 48 coli units per mgm.). Crystals were most readily obtained by concentrating the warm ethyl acetate solution to small volume and holding it overnight in the refrigerator. Culture fluids assaying 70 to 80 coli units per ml. permit the recovery of approx. 1.0 gm. per litre.

Properties of Crystalline Material

The re-crystallized substance melted at 112° C. and analysed as follows: C 54.82, 54.82; H 3.99, 4.01 (theoretical for $C_7H_6O_4$: C 54.55, H 3.93). It was found to reduce Fehling's solution and to decolorize permanganate. A 1-2000 solution in water showed no reduction in potency after being autoclaved for 15 min.

The bacteriostatic effect of the crystals on various species of bacteria as determined by the serial dilution method, using nutrient broth (total volume 5 ml., inoculated with 3 mm. loopful of 24 hr. culture; readings after 24 hr. at 37° C.) is shown in Table IV. Considerably greater antibacterial effect was noted against the Gram-negative species tested than against *S. aureus* with the exception of *P. aeruginosa*, which was considerably more resistant.

TABLE IV

BACTERIOSTATIC ACTION OF CRYSTALLINE CLAVIFORMIN PRODUCED BY TWO PENICILLIA ON VARIOUS ORGANISMS

Test organism	Highest dilution inhibiting growth (nutrient broth)	
	Claviformin from *Penicillium* No 4	Claviformin from *Penicillium* No. 08
Staphylococcus aureus 490 (*F.D.A.* 209)	120,000	120,000
Escherichia coli 117	300,000	240,000
Pseudomonas aeruginosa 499	10,000	<10,000
Eberthella typhosa 421	400,000	400,000
Salmonella typhi-murium 440	240,000	200,000
Salmonella paratyphi 232	300,000	200,000
Shigella alkalescens 498	200,000	120,000
Proteus vulgaris 497	300,000	200,000

Toxicity tests were made with crystals from *Penicillium* No. 4 and also with apparently similar crystals (m.p. 111° C.) obtained from culture No. 08. In both cases the lethal dose for mice was 0.1 to 0.2 mgm. by intraperitoneal, and 0.2 to 0.4 mgm. by subcutaneous injection. In these respects the action agrees well with that reported for claviformin, clavacin, and patulin.

Acknowledgments

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TREATMENT OF EXPERIMENTAL MOTION SICKNESS IN HUMANS[1]

By R. L. Noble[2]

Abstract

Experiments have been conducted on the production and treatment of motion sickness on human volunteers. Of 369 men tested after treatment by a placebo, 56.6% vomited. Tests were repeated at weekly intervals on 183 susceptible individuals so that 661 tests were performed. Repeat tests on 24 subjects not susceptible after treatment with a placebo showed that 12% were ill when no treatment was given. Ten susceptible subjects were swung through only one-half the usual degree of swinging. Of these eight vomited. This procedure selected persons of marked susceptibility to motion sickness.

The consistency of the time of vomiting was determined by 106 tests on 65 susceptible men. In repeated tests after a placebo only eight were 13 min. more than the control test. For assaying drugs a standard procedure was adopted. Susceptible individuals were classed as those who, after taking a placebo, vomited before 30 min. of swinging. A susceptible individual was considered protected if in a test a week later he did not vomit and remained swinging for 13 min. longer than his control time. Improvement was present if a person vomited but the time was 13 min. longer than the control. In no case was swinging less than 30 min.

The effect of a number of barbiturates on swing sickness has been determined. The most effective was *V*-12, ethyl-β-methylallylthiobarbituric acid, when administered in a divided dose of a total of five grains. In this case 78% were protected or improved. With a single dose of three grains 26% were similarly affected. *V*-17 (allylisoamylbarbituric acid)—two grains, *V*-16 (dicrotylbarbituric acid)—three grains, *V*-15 (allyl-*sec*-butylbarbituric acid)—one grain, *V*-14 (ethylcrotylthiobarbituric acid)—two grains, *V*-9 (*n*-butyl-1-methylallylthiobarbituric acid)—nine grains, and sodium amytal—one grain, showed less or no effect. Hyoscine hydrobromide in single doses of 0.4 and 0 65 mgm. showed 31 and 50% protected or improved respectively. The R.C.N. remedy gave a figure of 58%. After removal of nicotinic acid from the R.C.N. mixture 60% were benefited. Nicotinic acid alone, or pretreatment with thiamin gave no protection.

A combination of three grains of *V*-12 with hyoscine was more effective than when either drug was used alone. With a dose of five grains of *V*-12 and hyoscine the results were only slightly better (80%) when compared with the same dose of *V*-12 alone (78%). It is suggested that the most effective form of administration of *V*-12 may be by divided doses of two and one-half grains twice daily for at least 24 hr. previous to exposure to motion.

Previous papers (1, 2) have described some of the factors causing motion sickness and the methods used to test therapeutic measures in experimental animals. Tests on dogs in 1942 showed that certain drugs were highly effective in preventing motion sickness and it was of importance then to determine how they would act on humans. As previously pointed out, the study was begun in an effort to find barbiturates that might exert a specific action on the brain centres involved in motion sickness. Early experiments on animals showed that the protective effect of barbiturates was a specific type of action and not necessarily related to the hypnotic or anaesthetic

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properties of the compound. Furthermore, prevention of vomiting could be accomplished in 100% of susceptible animals with no untoward symptoms of the drug being noted. Many barbiturates were highly active, as will be described in a separate paper. The problem that arose, therefore, was to determine which barbiturates would be best tolerated by humans and would exert the most beneficial action. To select such a compound it was decided to perform therapeutic tests on human volunteers on the swing. Suitable compounds selected could then be tested on air or sea trials.

When the first barbiturates that protected dogs were described, a number of investigators had swings and facilities for conducting therapeutic tests on humans. Such compounds as *V*-1 (ethylallylthiobarbituric acid), *V*-5, (ethyl-1-methylbutylthiobarbituric acid), *V*-7 (ethylisoamylthiobarbituric acid), *V*-8 (ethyl-*n*-hexylthiobarbituric acid), and *V*-9 (*n*-butyl-1-methylallylthiobarbituric acid) were tested and have been included in various reports. No doubt these results will be published in the future. The present paper describes tests on humans performed at a later date when three electrically driven swings were available at McGill University. From a consideration of the results of experiments previously described on dogs, it was decided to adopt somewhat similar arbitrary standards for humans. The method of selecting susceptible individuals and that used for testing barbiturates will be described in the first part of the paper. Throughout this work it was never possible to have adequate numbers of volunteer subjects and many of the groups tested were too small to yield anything but suggestive results. The co-operation and help of the authorities in making it possible to obtain volunteers was greatly appreciated. Two groups of volunteers were tested, one from Reserve Army units in Montreal and the other of students of McGill University. These individuals are to be thanked for the inconvenience and discomfort that they allowed to make these experiments possible. Many of the tests had to be conducted in the evenings and the technical assistance rendered by Mr. A. Battista, then a medical student, was greatly appreciated. A study of the reliability of the swing test on motion sickness was made on the first 77 volunteers and was reported to the National Research Council by H. H. Jasper, A. Battista, M. Bornstein, and R. L. Noble (3). A continuation and extension of these observations form the basis of the standards for testing to be described in this paper.

The therapeutic measures to be discussed include a number of barbiturates and it is of interest to describe the rationale for their use. The first compound shown to be highly effective in dogs was *V*-1, referred to previously. This compound possessed only moderate potency as an hypnotic so that it was expected that tests on humans could be conducted without any side effects being present. Unfortunately, tests showed that the dose could not be increased beyond five grains without causing undesired reactions. A number of compounds were then tested that had very low hypnotic properties in animals, but were still quite active in protecting against sickness. Such examples were *V*-7, *V*-8, and *V*-9. When tested on humans, however, it was

found that even in doses up to nine grains little evidence of protection was seen. Apparently the dog was much more readily treated than the human. A new type of barbiturate was then introduced to get around some of the difficulties. It was found in animal tests that certain compounds were highly active against sickness but also had convulsant rather than hypnotic properties. The convulsant action was typically produced in rats. Such a compound, *V*-12, ethyl-β-methylallylthiobarbituric acid, was introduced in the hope that its protective action was powerful enough to act in humans and that its convulsant rather than hypnotic action would allow a sufficient dose to be given without side effects. Results with *V*-12 and other barbiturates will be described. For comparative purposes tests have been conducted with hyoscine and hyoscyamine mixtures and with a mixture introduced by the Royal Canadian Navy. The effectiveness of these drugs had previously been described by other workers.

Methods

Only the general methods used in the tests will be described here since the special conditions are included with the results. Three electrically driven swings designed by Major A. Cipriani, as previously used for animal tests, have been used to cause motion sickness. The individual to be swung sat in a special seat with back, head rest, and foot support. The head was slightly flexed and fixed so that a line from the external auditory meatus to the outer canthus of the eye was parallel to the floor. The individual's head was approximately 2 ft. above the platform of the swing (Plate I). The radius of the swing was 14½ ft. and it completed 15 complete to and fro swings per minute. For all comparative tests the swinging was through an angle of 90°. Swinging was continued for periods not shorter than 30 min. unless the individual vomited. The individual was urged to continue until he vomited, since this was the only criterion used for susceptibility, and all individuals were found to obtain symptomatic relief by vomiting. Volunteers were used for repeated tests; as far as possible three or four tests were performed on the same person. In some cases swinging was performed six times. Tests were made at intervals of one week in order to prevent adaptation. The time of day of each test for an individual was the same, so that the relationship to the taking of meals would be constant. It was impossible to isolate the men for the tests so that other individuals were often waiting for a short time in the same room as the swings. The swings were separated by a partition so that no person on the swing could see another individual vomiting. The men were told that most people became sick after swinging and that they were not to try and prevent vomiting. Each was given capsules before every test. In testing for susceptibility, placebos of lactose were used. The individual was told that the tests were to compare the effectiveness of different drugs, and that he would be given one on every occasion. In only one test to be described, no therapy was administered.

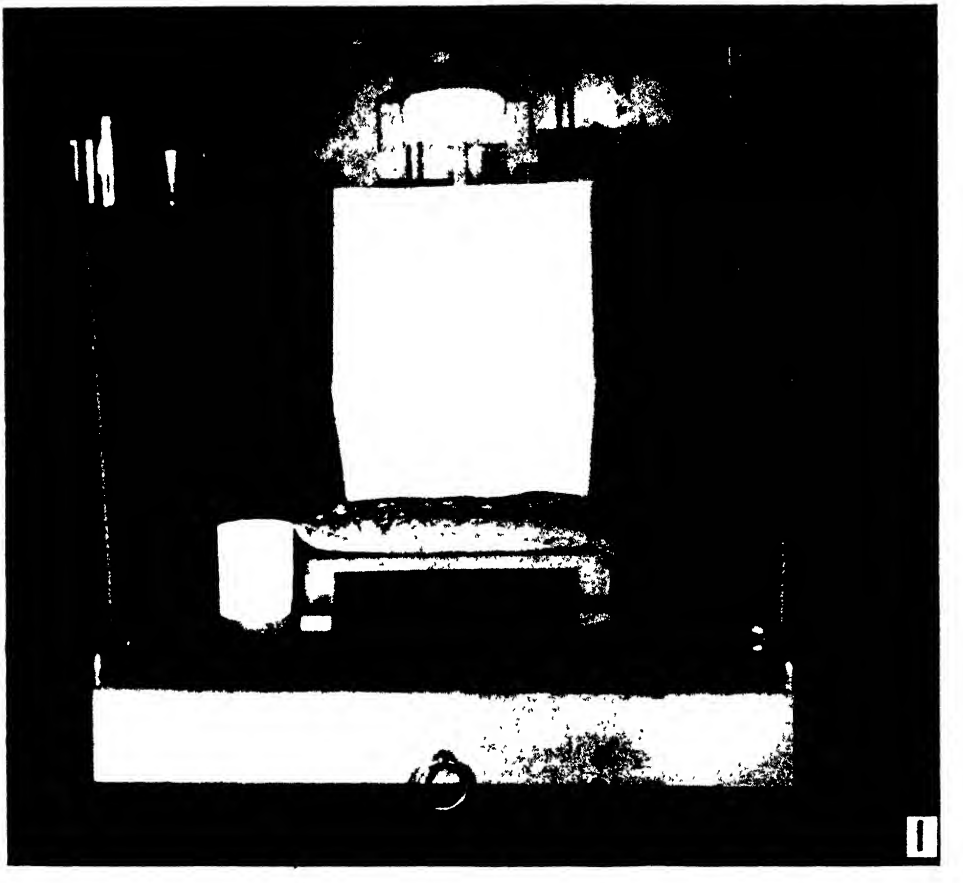

FIG. 1. *Motion sickness swing.*

Results

Incidence of Sickness

For all the experiments it was possible to test 109 army men and 260 students. Of these 60 and 55%, respectively, vomited and were classed as susceptible to motion sickness. Taken together out of the group of 369 men, 209 were ill, giving an incidence of 56.6%. Of the susceptible individuals 26 dropped out after the first test. The remaining 183 men were swung 661 times, 14 on six occasions, 10 on five, 84 on four, 42 on three, and 33 only twice. The incidence of vomiting was recorded in different age groups. Of the students 110 were under 20 years of age and 53% were susceptible. The army group showed a greater range of ages so that of 28 men from 18 to 29 years of age, 60% were susceptible. From 30 to 39 years, of 51 men 53% vomited. Of 23 men over 40 the incidence was 74%. This last figure suggests that the frequency of swing sickness may increase after 40 years of age.

Some experiments were performed on the non-susceptible students. These individuals had completed 30 min. on the swing after treatment with placebo in their first test. In one experiment 70 students were given a placebo in a pink capsule for their first test—of these 50% vomited. The main group with ordinary white placebo gave a susceptibility rate of 57%. This test was run at a time when the protective action of pink capsules against seasickness was widely publicized. Twenty-four students were swung again a week later but without any treatment. Of these three vomited on the second test. This would suggest that 12% may have been protected by placebo treatment. On the other hand it may represent the number of individuals whose time of vomiting varies from 25 to 35 min. Of the group re-tested without treatment, 11 of them had received a pink placebo the first time. One of these vomited on the second test. The psychological effect of treatment with placebo in these tests therefore could not be greater than 12%. The use of pink placebos did not enhance their value.

In initial tests an attempt was made to relate susceptibility on the swings to any previous history of motion sickness. The use of a questionnaire was found unsatisfactory and it was not possible to obtain details of the previous history from every individual. From general questions, however, one conclusion was drawn. Men who had had considerable experience at sea in rough weather, and a few who had flying experience, and who stated that they were quite immune, were frequently sick after only short periods of swinging. In other words, the motion of the swing seemed a much more effective stimulus in these cases. This discrepancy in susceptibility between seasickness and swing sickness seems the same as that described for dogs in a previous paper (1). Individuals who had definite histories of motion sickness in boats, cars, or on devices in amusement parks, were practically always made sick by swinging.

Various workers have attempted to use swings for the purpose of preselecting susceptible individuals so that they could be suitably placed in the various branches of the armed services. In general, these tests have not given an

indication as to how an individual would later react to motion at sea or in the air. From the observations mentioned above, it was believed that the swings used created too severe a test and so would tend to classify too many individuals as susceptible. Furthermore, an incidence of 56.6% sickness on the swings after 30 min. is obviously a high rate when compared with figures for seasickness. The observation (1) that the most susceptible dogs could be made ill when swung through an angle as small as $22\frac{1}{2}°$ suggested that humans might be tested with reduced swinging. Ten subjects who seemed especially susceptible to the full swing were selected, and were then swung through an angle of 30° instead of 90°. This was the greatest reduction in swinging that could be accomplished without having to shut off the electric-motor–driving mechanism. With the reduced swing 8 of the 10 men vomited, the two who did not were very nauseated. The average times of vomiting were $10\frac{1}{2}$ min. for the full swing, and $14\frac{1}{2}$ min. on the reduced swing. This result suggests that, as with dogs, it is possible to select highly susceptible individuals by using reduced swinging. One would expect that such persons would be the ones who would show sickness from other forms of motion. From the services point of view preselection of such cases by swing tests might prove to be of value.

Consistency of Results

At the time when the therapeutic tests were started on humans no standard technique in use appeared entirely satisfactory. In some reports susceptible individuals were classed as those who vomited, felt nauseated, or asked to have the swing stopped. These 'susceptibles' were then re-tested after a week or less following treatment. If the individual was now swung for 30 min. without vomiting or with less symptoms he was classed as protected or improved. In many cases the time on the swing was the same or only a few minutes longer than in the initial test with the placebo. An attempt was made to incorporate more rigorous standards and to determine the reliability from repeated swing tests. A report was made on 77 men (who were swung twice at an interval of one week), after placebo treatment (3). A susceptible individual was classed as one who vomited or felt severely ill in 30 min. Of this group 39, or 51%, were susceptible in both tests. Six men were susceptible on the first test but not on the second, whereas three men were susceptible only on the second test. If one considered only the first test there were 45 men, or 58.5%, classed as susceptible. Of these, six, or 13.5%, would not have been susceptible on a second test or would have given an erroneous test if used for therapeutic experiments. When the times of vomiting for the two swingings were compared it was found that six men were able to stand the second swinging for 10 or more minutes longer than they did the first swinging.

A continuation of this study was made on 65 individuals. These were selected after a single treatment with placebo but vomiting was used as the only criterion of susceptibility. They then were re-tested once or more often,

with placebo, at weekly intervals. In all, 106 tests were conducted. The times of vomiting compared with those found in the initial test may be seen in Fig. 2.

It may be noted that the times of vomiting are fairly close together, so that 54% of the repeat tests were within ± four minutes of the initial time. In only 7.5% of tests did individuals go more than 13 min. of their control time. In 124 students the average time of vomiting was 15.7 min.; 27% showed times of over 20 min. Similarly, of the army group of 66 men the average time was 15.2 min. and 20% were more than 20 min.

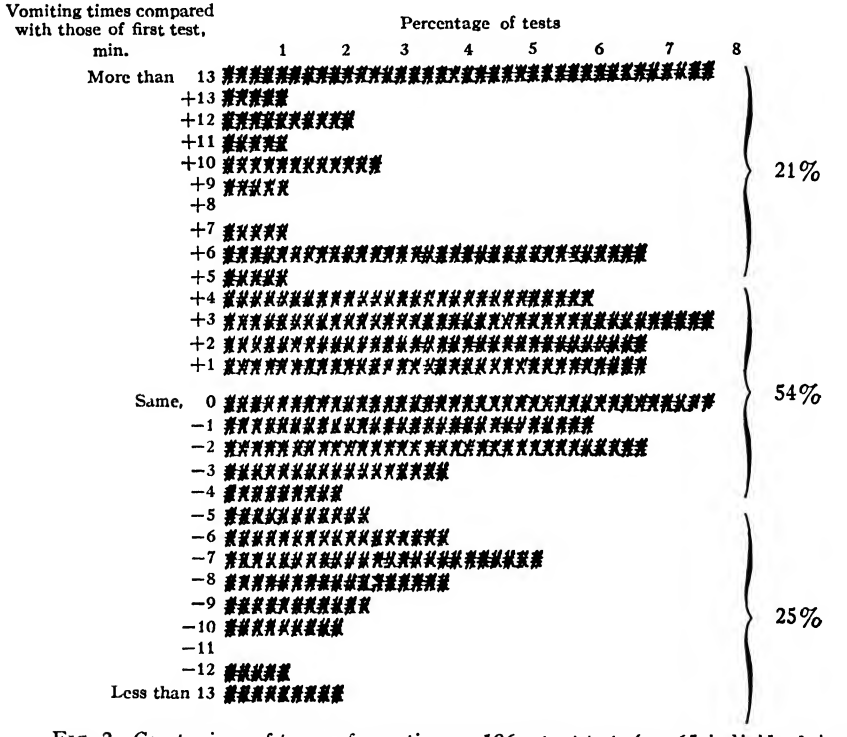

FIG. 2. *Comparison of times of vomiting in 106 repeat tests (on 65 individuals) with time found in initial test.*

On the basis of these tests a provisional standard technique was adopted for therapeutic tests and may be briefly stated. The swing used was of the dimensions described and swinging was through an angle of 90°. An individual was tested for susceptibility after being treated with a placebo. Anyone who vomited within 30 min. was classed as susceptible. Further tests were conducted at weekly intervals. After treatment with the various drugs to be tested any individual was considered to be protected if he did not vomit after swinging was continued for at least 13 min. longer than in the control test. In no case was swinging less than 30 min. Individuals were listed as improved if they continued on the swing for 13 min. more than their control test even though they eventually vomited. By using this standard it was

found that seven men had been sick on each of six swing tests. Two tests were with placebo, three after treatment with inactive drugs, and one after treatment with an active compound. Another group of five men had been swung six times. The final two tests in this case were with active compounds, one of which effectively prevented vomiting. The time of vomiting was prolonged after treatment with the other active drug. These results give some indication of the consistency of the times of vomiting in repeated tests, and are listed in Table I.

TABLE I

TIMES OF VOMITING IN REPEATED TESTS

Volunteer No.	Treated with placebo		Treated with inactive compounds			Treated with active compounds
	Vomiting times, min.		Vomiting times, min.			Vomiting time, min.
1	5	4	6	4	9	12
2	13	8	8	6	6	11
3	9	9	2	5	6	6
4	15	17	19	12	9	14
5	13	13	13	11	7	8
6	17	15	21	18	9	25
7	7	10	17	12	4	7
1	6	12	17	8	—	22 (30 protected)
2	19	26	17	9	—	17 (30 protected)
3	6	18	12	4	—	24 (30 protected)
4	18	24	20	10	—	31 (38 protected)
5	18	17	17	12	—	35 (32 protected)

These times of vomiting may be seen to be relatively consistent except where prolongation occurred after treatment with compounds known to be active. These individuals, however, probably represent a highly susceptible group and, from a consideration of the results obtained on dogs (2), one might anticipate consistent results.

Effects of Barbiturates on Swing Sickness

Therapeutic tests were first begun using volunteers from the Reserve Army. At this time no standard procedures had been worked out, so that these tests are listed separately. Effective compounds in these cases were ones that prevented vomiting in 30 min. in susceptible subjects, irrespective of the control time for vomiting. Some of these individuals could be classified as protected or improved under the new conditions, and they are listed separately for comparative purposes. The barbiturates tested were *V*-12 (ethyl-β-methylallylthiobarbituric acid) and its sodium salt, *V*-9 (*n*-butyl-1-methylallylthiobarbituric acid), *V*-15 (allyl-*sec*-butylbarbituric acid), and *V*-14 (ethylcrotylthiobarbituric acid). The doses used were three, nine, one, and two grains, respectively. Benzedrine sulphate (10 mgm.) was given at the same time as *V*-9 and *V*-15 to reduce hypnosis. The drug was given approximately three hours before the swing test. The results are listed in Table II.

TABLE II

EFFECTS ON MOTION SICKNESS OF TREATMENT WITH BARBITURATES

Barbiturate No.	Dose, grains	No. of volunteers	'Protected', %	(New criteria)	
				Protected, %	Improved, %
V-9	9	10	10	0	0
V-15	1	19	37	29	0
V-14	2	19	37	25	0
V-12 acid	3	27	37	26	22
V-12 sodium salt	3	20	35	20	0

These results indicate that *V*-9 was not effective in the dose used. *V*-15, *V*-14, and *V*-12 had definite protective action. When judged by the new criteria, *V*-12 acid seemed the most potent compound. Benzedrine, which was added to *V*-9 and *V*-15, was obviously without effect on motion sickness. Before the swing test each individual was questioned as to whether or not any side effects of the drug had been noticed. No effects of *V*-9 were found. With *V*-15 and benzedrine, five men stated that they felt light-headed or sleepy, four complained of a drunken feeling or dizziness. After *V*-14, four men had mild complaints of sleepiness or a light-headed feeling. With *V*-12, three men felt slightly sleepy and three complained of dizziness. All these symptoms were classed as mild and led to no real discomfort or incapacitation.

The results to be presented in the remainder of this paper were obtained chiefly on student volunteers and all the tests have been conducted in the standard manner described above. The results should therefore be directly comparable. Some other barbiturates were included in these tests, namely, *V*-16 (dicrotylbarbituric acid), and *V*-17 (allylisoamylbarbituric acid), and sodium amytal. These were given as single doses of three, two, and one grain, as previously described. Tests on *V*-12 were again repeated and the *V*-12 acid was also given with hyoscine hydrobromide, 0.4 mgm. These results have been calculated in a different manner as they were designed to determine the value of the *V*-12 hyoscine mixture and of its component parts. The susceptible individual, therefore, was first tested with the mixture. If he was protected he then received in the next tests the hyoscine or *V*-12 alone. If the mixture did not protect, the subject was retested but with a higher dose, namely, five grains, of *V*-12 with hyoscine. In such a case the *V*-12 was given in two doses; the initial dose of three grains was given with breakfast, the second dose of *V*-12 with lunch—some three to five hours before swinging. The hyoscine was taken separately one and one-half to two hours before the test. In evaluating these tests it has been possible by inference to increase the numbers for the test. Thus it has been presumed that an individual who was protected by three grains of *V*-12 and hyoscine would also have been protected by the larger dose of five grains and hyoscine. In estimating the effects of five grains of *V*-12, therefore, those protected by

three grains are also included. Similarly, for an individual who was not protected by *V*-12 and hyoscine, it could be assumed that the same dose of hyoscine alone would not be effective. Had sufficient numbers of men been available for testing, this procedure would not have been necessary. However, it is believed that the values expressed serve as an indication of the relative effectiveness of the various compounds. It should be pointed out that no evidence of any synergistic action or of one compound inhibiting the effect of another has been noted in any tests. These results are shown in Table III.

TABLE III

THERAPEUTIC TESTS WITH BARBITURATES

Compound No.	Dose, grains	No. of tests	Protected, %	Improved, %	Total, %
V-16	3	16	0	6 5	6 5
V-17	2	15	6.7	13 3	20 0
Amytal	1	16	6 5	6 5	13.0
V-12	3	30	23	3.3	26 3
V-12	5	23	65	13	78.0
V-12 + hyoscine HBr.	3 0 4 mgm.	35	40	8 5	48.5
V-12 sodium salt + hyoscine Hbr.	3 0 4 mgm.	12	33	8 5	41.5
V-12 + hyoscine HBr.	5 0 4 mgm.	30	70	10	80.0

These results show that in the doses used *V*-16 and sodium amytal had little effect on motion sickness. *V*-17 apparently had some protective action. *V*-12 alone was effective in a dose of three grains, to about the same extent as found previously, and as shown in Table II. When the dose was raised to five grains, the protective action was very marked. In this case, as previously noted, the dose was divided. It is believed that this may be an important factor in increasing the effectiveness of this compound. When *V*-12 was combined with hyoscine it was definitely more effective in the dose of three grains, but only slightly better when the dose of five grains was used. This effect might be anticipated when the results of hyoscine alone are considered in the following section. A small number of men were given *V*-12 as the sodium salt with hyoscine. Apparently the effectiveness was about the same as when the acid was used. Despite this result the impression has been formed that the acid is the more effective and better tolerated form of this compound. Side reactions from the compounds used were again slight and transitory. No inconvenience or incapacitation in any way was experienced. A few highly susceptible individuals were given *V*-12 in three doses for a total of six or seven grains. These were given with each meal and the test was performed in the evening. In 5 of 10 tests protection or improvement took place, which was approximately the expected result from a dose of five grains. From some recent observations on cases of motion sickness it appears

that pretreatment of 24 hr. may give optimal results. In such cases a dose of two and one-half grains was given with a meal in the morning and afternoon. Taking *V*-12 with food reduces any side effects and probably prolongs its time of absorption. As will be pointed out in a separate paper, large daily doses in animals may tend to be cumulative. If *V*-12 is to be used for prolonged periods, therefore, it is advisable to have some supervision.

Effects of Hyoscine, Hyoscyamine, and Vitamins on Motion Sickness

Comparative tests have been made using hyoscine alone and in mixtures and using nicotinic acid and thiamin. These tests were made subsequent to reports on their efficacy by other investigators. Hyoscine hydrobromide has been administered alone in a dose of 0.4 mgm. or 0.65 mgm. given one and one-half to two hours prior to the test. A mixture of hyoscine hydrobromide (0.3 mgm.), hyoscyamine hydrobromide (0.8 mgm.), and nicotinic acid (150 mgm.) was tested as such, as was a combination of the first two ingredients without the nicotinic acid. The nicotinic acid was enclosed in an enteric capsule. The former mixture, put up in pink capsules, was developed by personnel of the Royal Canadian Navy Research Unit, Toronto, and was kindly supplied by them. It is referred to as the R.C.N. remedy. A modified form of this mixture was also tested. It was composed of hyoscine hydrobromide (0.3 mgm.), hyoscyamine hydrobromide (0 6 mgm.), nicotinic acid (150 mgm.), and sodium amytal (65 mgm.), and is referred to as R.C.N.-amytal. These various mixtures were administered from two and one-half to three and one-half hours before the test. Nicotinic acid was tested alone in a dose of 100 mgm. given 15 min. before swinging. A few individuals were treated with thiamin hydrochloride. This was given in a dose of 15 mgm. daily for two days before and on the day of testing. The volunteers were arranged in groups so that comparisons of certain mixtures could be made on the same individuals. It was possible to have 10 men treated by hyoscine (0.65 mgm.), R.C.N., and R.C.N.-amytal, on three successive tests. Similarly, another group, of 23 men, received R.C.N. and R.C.N. without niacin. The results obtained with these groups are listed separately, as well as the complete results, in Table IV.

It may be seen from the table that many of the treatments used were quite effective in preventing sickness. Hyoscine alone was definitely active, especially in the larger of the doses used. The R.C.N. mixture was the most potent combination and it may be seen that removal of the niacin did not alter the results. Similarly, in the comparative test on 23 men the mixture without niacin was slightly more active. Niacin when tested alone was inactive; in fact the average time of vomiting was definitely less than for the control, suggesting that this substance may have an adverse action. This result may be seen in Table V. R.C.N.-amytal was effective, but slightly less so than the R.C.N. mixture. In the group of 10 men tested with hyoscine, R.C.N., and R.C.N.-amytal, the number protected was the same although the number improved varied. Thiamin was found to be ineffective. A relation between the results with hyoscine and the R C.N. mixture could be

TABLE IV

THERAPEUTIC TESTS WITH HYOSCINE, R.C.N. REMEDY, ETC.

Treatment	Dose, mgm.	No. tests	Protected, %	Improved, %	Total, %
Hyoscine HBr.	0 4	48	29.0	2 1	31.1
Hyoscine HBr.	0 65	28	50.0	50.0	50 0
R.C.N.	—	43	51 0	7.0	58 0
R C.N. without niacin	—	25	40 0	20 0	60.0
R.C.N.-amytal	—	22	41 0	13 5	54 5
Nicotinic acid	100	17	0	0	0
Thiamin	15 (daily)	5	0	0	0
Tests on same individuals					
Hyoscine HBr.	0 65	10	60	0	60
R.C.N.	—	10	60	10	70
R.C.N.-amytal	—	10	60	20	80
R.C.N.	—	23	43 5	5	48 5
R.C.N. without niacin	—	23	39.0	17.5	56 5

TABLE V

INDIVIDUAL TIME OF VOMITING AFTER VARIOUS TREATMENTS

Volunteer No.	Placebo 1st	Placebo 2nd	*V*-12 3 gr *	*V*-14 2 gr	*V*-15 1 gr.	*V*-16 3 gr.	Niacin 100 mgm	Hyoscine 0 65 mgm.	(Reduced swing)	*V*-12 2 gr. repeat.
1	26	29	(33)	—	(41)	28	11	—	—	—
2	22	24	(30)	—	(35)	25	19	—	—	—
3	12	13	(30)	—	—	—	11	—	10	14
4	6	12	(30)	—	17	22	8	—	—	—
5	17	10	(30)	—	12	—	8	—	—	—
6	19	26	17	(30)	—	17	9	—	—	—
7	6	18	24	(30)	—	12	4	—	—	5
8	18	24	31	—	(38)	20	10	—	—	—
9	5	4	12	9	—	6	4	—	—	—
10	13	8	11	—	8	6	6	—	—	—
11	9	9	6	6	—	5	2	—	—	—
12	15	17	14	19	—	12	9	—	—	—
13	13	13	8	—	13	11	7	—	—	—
14	17	15	25	—	21	18	9	—	—	—
15	7	11	—	—	5	—	3	—	—	—
16	7	10	7	—	17	12	4	—	—	—
17	18	17	25	—	17	—	12	—	—	—
Average	14	15	16	11	14	15	8			
18	15	—	(30)	—	—	—	—	(30)	(30)	27
19	25	—	(30)	—	—	—	—	(40)	17	—
20	14	—	12	—	—	—	—	(30)	19	9
21	7	—	10	—	—	—	—	(30)	28	19
22	10	—	12	—	—	—	—	16	16	—
23	3	—	4	—	—	—	—	17	11	4
24	8	—	8	—	—	—	—	8	4	10
25	17	—	(30)	—	—	—	—	19	—	—

Note.—Time in parentheses indicates men did not vomit.

* *Gr. = grains.*

worked out by assuming that hyoscyamine possessed three-fifths of the activity of hyoscine. If one disregarded the other components of the mixtures it was found that the results fell roughly on a line in proportion to their calculated hyoscine content. In this case the niacin or amytal did not exert any beneficial effect.

The side effects noted in these tests were slight and transitory. Most men receiving the larger dose of hyoscine and the various R.C.N. mixtures complained of dryness of the mouth. In many cases this was pronounced but only caused some inconvenience. Two individuals complained of blurring of vision. After nicotinic acid about one-third of the men had some blushing of the face. This was not noted when the drug was given in the enteric capsules. Many of the individuals who were protected stated that they had a numbness of the stomach and that the unpleasant sensations experienced in their control test were prevented. Those protected by *V*-12 did not have this curious numb sensation of the stomach.

If these results are compared with those of Table III it is found that the most efficient remedy tested was the larger dose of *V*-12 and hyoscine, giving 70% protection, although *V*-12 alone was approximately as active, protecting 65%. The R.C.N. remedy protected 51% and the larger dose of hyoscine protected 50%. These were the next most active compounds. A mixture of the lower dose of *V*-12 and hyoscine was more effective than either drug alone. In this case some individuals were protected by one and not by the other. With the larger dose little difference was noted, since most individuals were then protected by both drugs. In tests on dogs it has been found that hyoscine has no protective action against motion sickness. This would suggest that the action of hyoscine and barbiturates are probably of a different nature. Theoretically, therefore, a mixture might be expected to be the most effective form of therapy. As previously stated, increased effectiveness might be found if pretreatment with *V*-12 could be commenced 24 hr. before the exposure to motion. It is believed that the single doses used in these experiments have been the maximum that would be tolerated without giving rise to an undesirable number of side effects.

In order to give some indication of the typical results obtained after treatment on the same group of individuals, a protocol showing the full details of a set of experiments on 25 men has been listed as Table V.

In Table V may be seen the type of results that were obtained and reported in this paper. In some cases it may be noted that certain individuals were protected by one barbiturate but not by another. The average times of vomiting of the first 17 men has been listed. The times may be seen to be very constant except in the case of treatment with niacin. As referred to above, it seemed that this substance caused the men to vomit more rapidly.

Acknowledgments

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THE EFFECT OF RIBOFLAVIN DEFICIENCY ON RENAL DAMAGE DUE TO SULPHANILYLGUANIDINE IN THE ALBINO RAT[1]

By John A. Ziegler[2]

Abstract

Certain intestinal bacteria can synthesize some of the B vitamins, and others may synthesize vitamin K. In an attempt to deprive rats, maintained on a riboflavin deficient ration, of riboflavin that might have been synthesized in the intestine, sulphanilylguanidine (sulphaguanidine) was fed with the diet at a level of 0 75% for 78 days. Four groups of animals were used. The control group was maintained on the basal ration; Group 2 on the basal ration plus 0 75% sulphaguanidine; Group 3 on the riboflavin deficient ration; and Group 4 on the riboflavin deficient ration plus 0 75% sulphaguanidine. Growth curves, clotting times, red cell counts, and sulphonamide concentrations in blood, urine, and faeces were determined and chemical analyses of urinary calculi were performed. In addition, microscopic examinations of liver, spleen, kidney, and cornea were made in each animal. Sulphaguanidine added to the basal ration had a negligible effect on growth, but when added to the riboflavin deficient ration a cessation of growth occurred accompanied by anaemia and hypoprothrombinaemia The symptoms of vitamin K deficiency disappeared upon administration of 2-methyl-1,4-naphthoquinone. Renal damage was observed in every rat that had received sulphaguanidine and was especially severe in the animals comprising Group 4 Urinary calculi were found only in those animals that had received sulphaguanidine and the incidence was highest in Group 4. The data indicate that the symptoms of riboflavin deficiency can be aggravated by sulphaguanidine and conversely that the toxic effects of sulphaguanidine may be aggravated by riboflavin deficiency.

Introduction

Sulphanilylguanidine (sulphaguanidine) has found rather widespread clinical utility as an intestinal antiseptic agent. Although like sodium sulphanilylsulphanilate, N′-(2-hydroxyethyl) sulphapyridine, N′-diglycolsulphapyridine, and others, sulphaguanidine is appreciably water soluble, it is but poorly absorbed from the intestine. This property permits a high concentration of the sulphonamide to be maintained in the intestinal tract with a coincident low blood concentration of the drug due to prompt excretion by the kidneys.

It is known that certain normally present intestinal bacteria are able to synthesize some of the B vitamins (2), and that others may synthesize vitamin K (10, 12). It had been observed that weanling rats placed on our riboflavin deficient ration grew too rapidly in the supposed absence of riboflavin, and it was suspected that a significant amount of refection had occurred. Firor and Poth (5) have shown that in patients whose intestinal mucosa was intact, the administration of adequate doses of sulphaguanidine nearly always resulted in a profound decrease in the number of intestinal coliform bacteria. It was therefore decided to attempt to inhibit the intestinal synthesis of riboflavin

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Contribution from the Department of Pathological Chemistry, Banting Institute, University of Toronto, Toronto, Ont. The research was supported by a grant from the National Research Council, and this paper represents an excerpt from a thesis submitted to the University of Toronto in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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in rats by chronic dosage with sulphaguanidine. In a preliminary experiment several rats were placed on a riboflavin deficient ration containing various percentages of sulphaguanidine and the growth curves were determined. Slowing and cessation of growth were indeed obtained, but those animals that had been maintained on the ration containing 2% of the sulphonamide died in three to four weeks. At autopsy, all the animals showed urinary calculi that were found to contain a high percentage of sulphonamide. Renal damage was severe in all cases and several animals displayed complete hydronephrosis. Several rats maintained on the ration containing 1% of the sulphonamide died, while there were no deaths when sulphaguanidine was fed at the 0.5% level.

It was therefore decided to fix the concentration of the sulphonamide in the ration at 0.75%.

Methods

Weanling Wistar strain albino rats of mixed sexes were placed on various diets, as indicated below, for a period of 78 days. All diets consisted of modifications of the basal ration.

Basal ration (Diet 1):

Dextrin	72	Salt mixture	4
Labco casein	18	Alpha tocopherol	0.3
Lard	3	Oleum percomorpheum	0 1
Corn oil	3	Vitamin supplement*	

* *Vitamin supplement for each kgm. of basal ration.*

Choline chloride	*2 0 gm.*	*Pyridoxine*	*3 0 mgm.*
Niacin	*50 0 mgm.*	*Thiamin*	*3 0 mgm.*
Pantothenic acid	*10 0 mgm.*	*Riboflavin*	*3 0 mgm.*

Diet 2: Basal ration + 0.75% sulphaguanidine.

Diet 3: Basal ration without riboflavin.

Diet 4: Diet 3 + 0 75% sulphaguanidine.

Group 1

Five males and five females, all litter mates, were placed on Diet 1 at 21 days of age. For the duration of the experiment each rat was weighed weekly, and red cell counts and clotting time determinations were made at three 26 day intervals. After 78 days on the diet the rats were anaesthetized with nembutal and the eyes, liver, spleen, and kidneys were removed for microscopic examination.

Group 2

Five males and five females, all litter mates, were similarly placed on Diet 2 at 21 days of age. Weights were taken each week, red cell counts, clotting times, and blood sulphaguanidine levels were all determined at 26-day intervals. In addition, at seven-day intervals, three males and three females were kept in metabolism cages and 24-hr. specimens of urine and faeces were collected for sulphaguanidine determinations. At the termination of the

experiment, the animals were anaesthetized with nembutal and the eyes, liver, spleen, and kidneys were removed for examination.

Group 3

Five males and five females were placed on Diet 3 and were treated in the same manner as those in Group 1.

Group 4

Seven males and seven females were placed on Diet 4 and were treated in the same manner as those in Group 2. During the experiment two males and one female from Group 4 died and were discarded after autopsy in order not to confuse the microscopical findings with post-mortem changes.

Clotting times ("prothrombin times") were determined by the method of Ziffren *et al.* (14), the red cell counts by the usual haemocytometer method. Free and acetylated sulphaguanidine were determined in the blood and in the urine by a micro modification of the method of Bratton and Marshall (1, 9). Blood samples were taken directly from the hearts of the anaesthetized animals. In cases where urinary calculi were found at the conclusion of the experiment, the stones were removed, dried, weighed, and finely ground. Sulphaguanidine determinations were made and the murexide, xanthine, phosphate, and carbonate qualitative tests were performed whenever possible.

Sections were made from the livers, kidneys, and spleens, and the corneas were dissected from the eyes and stained by the method of Ziegler (13).

Results

The weight gains for the four groups of animals for the 78 day period of the experiment are shown in Table I. Each gain represents the average for

TABLE I

AVERAGE GAINS IN WEIGHT DURING 78 DAYS

Group	Net weight gain, gm	
	Males	Females
1	163	150
2	149	131
3	100	82
4	24	19

all the animals in each group. It will be observed that although the animals grew satisfactorily on the basal ration, when riboflavin was withdrawn, a reduced growth resulted, and when sulphaguanidine was added to the riboflavin deficient ration a cessation of growth occurred, to be followed by a severe premortal loss in weight. Sulphaguanidine added to the basal ration had a negligible effect on growth.

It is interesting to remark that the animals in Group 4, in contrast to those in other groups, showed a marked hyperexcitability and fierceness, resisting by biting any attempt to handle them. There was also a late muscular weakness, especially of the flexors of the hind limbs. Two of the males and three of the females of Group 3 showed patches of denudation bilaterally symmetrical and typical of riboflavin deficiency, but none of these showed any gross ocular changes. All the animals in Group 4 on the other hand showed marked denudation of the body and limbs, and in particular, areas of denudation 6 to 8 mm. in diameter about the eyes. Bloody, serous exudates had dried about the eyes and at the nasal openings. During the few instances when the rats were handled for taking the blood samples, one had the impression that the body temperature was lower than that of the other groups, although unfortunately it was not actually determined.

The red blood cell counts of Groups 1, 2, and 3 were but little affected by the diets on which the respective groups were maintained.

Group 4 on the contrary showed a very marked decrease in red cell count and a wide variation in size and staining of the cells indicative of a fairly severe anaemia. One male (Rat 34) and one female (Rat 41) animal withdrawn from the diet at 78 days and placed on the basal ration with the addition of 3 mgm. of 2-methyl-1,4-naphthoquinone per kgm. of diet for a further 30 days had nearly normal red cell counts at the end of this time.

As with the red blood cell counts, the clotting times ("prothrombin times") in Groups 1 and 3 remained sensibly unaltered throughout the experiment. The clotting times ("prothrombin time") for Group 2 changed from an average of 38 sec. at the start to 54 sec. at 78 days, The results obtained by the method employed were so variable that the significance of this increase is doubtful. In Group 4 the clotting time increased from 36 sec. at the start to 92 sec. at 78 days and it is felt that this is a significant change. Rats 34 and 41 (of Group 4) after being placed on the basal ration plus 2-methyl-1,4-naphthoquinone for 30 days showed a return to 39 sec. of the clotting time. It is therefore likely that a condition of hypoprothrombinaemia was induced by depriving the animals of the vitamin K normally synthesized by the intestinal bacteria. The anaemia observed in Group 4 is in accord with the work of others (11, 6) who have reported granulocytopenia and anaemia in rats maintained on diets containing different sulphonamides, and that these symptoms were due to a lack of folic acid in the ration (3).

The most commonly observed gross lesions were those of denudation in Groups 3 and 4, the accumulation of serous exudates about the nose and eyes of the animals in Group 4, and haemorrhages into the stomach in 40% of Group 4. These pathological changes were not observed in any of the animals of Groups 1 and 2.

The microscopic findings were as follows. The spleens in all groups showed relatively little change. There was a small amount of haemosiderin deposited in the Kupffer cells of the livers of the animals in Group 4. Of the rats on the riboflavin deficient ration (Group 3), the corneal lesions of ariboflavinosis

were observed in but 50%. It is true that nearly all the rats in this group showed some degree of limbic congestion, but well-marked corneal vascularization in which all the vessels were blood-filled was seen in only 5 out of 10 rats. By contrast, all the animals in Group 4 showed pronounced limbic congestion and corneal vascularization.

Renal damage of some degree was observed in every animal that had received sulphaguanidine. The kidneys of Groups 1 and 3 were essentially normal in every case. Those of Group 2 showed changes varying from a slight cloudy swelling to a slight degeneration of the cells of the convoluted tubules. All of the kidneys of the animals of Group 4 showed appreciable degeneration, including cloudy swelling and marked desquamation of the cells lining the convoluted tubules, hyaline droplets in the cells of the tubules, engorgement of the blood vessels, casts in the lumens of the tubules, extravasation of red blood cells into Bowman's capsule and proximal tubules, and increased interstitial fibrous tissue in the cortex. Several kidneys were almost completely necrotic and hydronephrosis was observed in 10% of the animals. The microscopic findings are summarized in Table II.

TABLE II

SUMMARY OF MICROSCOPIC FINDINGS

Group	Percentage of rats showing							
		Renal damage						
	Haemosiderin in Kupffer cells	Cloudy swelling	Degeneration of cells of conv tubules	Engorgement of blood vessels	Casts and RBC* in tubules	Increased interstitial fibrous tissue in cortex	Hydronephrosis	Necrosis
1	0	0	0	0	0	0	0	0
2	10	60	50	30	0	10	0	0
3	0	0	0	10	0	0	0	0
4	20	100	80	65	40	40	10	30

* *RBC = red blood cells.*

Urinary calculi were found in many cases, but only in those animals that had been fed sulphaguanidine. Calculi were not observed in either Group 1 or Group 3. The chemical analyses of the calculi are collected in Table III.

Discussion

Coprophagy has been observed to occur in our animal colony. This practice undoubtedly accounts for the unexpected growth of animals maintained on a riboflavin deficient ration since riboflavin in appreciable concentrations has been found in the faeces of animals maintained on this ration. Even the use of coarser screens in the animal cages fails to remove entirely the possibility of refection.

Escherichia coli has been shown to synthesize riboflavin *in vitro* (2) and we have demonstrated the presence of this coliform bacterium, among others,

TABLE III

SUMMARY OF CHEMICAL ANALYSES OF CALCULI

Group	No. rats with urinary calculi	Weight of calculi, mgm	Chemical analyses*					
			Free sulphaguanidine, %	Acetyl sulphaguanidine, %	Total phosphorus, %	Qualitative tests		
						Murexide**	Xanthine**	Carbonate**
2	4	3 5	20	6.4	46	+	−	−
		1 4	17	6 5				
		6 8	12	3 0	22	+	−	−
		7 2	23	8 1	29	−	−	−
4	7	2 0	13	4 7	17			
		1 3	19	5 0				
		1 5	7 1	1 6	+			
		6 1	24	3 7	14	+	+	−
		5 3	26	8 4	31	−	−	+
		3 3	11	4 2	32	+		
		8 6	14	4 9	26	+		

* *Tests were not performed when space is left blank.*

** + *Indicates a positive qualitative test;* − *indicates a negative qualitative test.*

regularly in the faeces of the rats maintained either on the basal or on the riboflavin deficient rations. For this reason, it is probably impossible, by means of a deficient or riboflavin free ration, to obtain an absolute degree of riboflavin deficiency since the rat absorbs appreciable quantities of intestinal riboflavin of bacterial origin.

The use, however, of a bacteriostatic agent in concentrations sufficient to inhibit the growth of riboflavin-synthesizing bacteria in the intestinal tract permits a new approach to the problem of riboflavin deficiency. It is necessary, of course, for the inhibiting agent to be present in concentrations that will cause bacteriostasis without being toxic to the host. No claim is made that such a condition obtained in this experiment. Indeed, in view of the high incidence of renal damage, it is more than likely that toxic concentrations of the sulphonamide existed. The ideal would be to establish and maintain a concentration of the sulphonamide in the intestine just sufficient to provide a stool free of riboflavin synthesizing organisms. The sulphonamide requirement for this condition would likely increase with the animal's age and with the ability of the riboflavin synthesizing bacteria in the intestine to produce sulphonamide resistant mutants. This is under investigation at present. That inhibition of riboflavin synthesis in the intestine had actually occurred was proved by the fact that the animals in Group 4 excreted about 1/10th as much faecal and urinary riboflavin as the animals in Group 3. Later experiments with rats on Diet 4 indicated that the count of coliform organisms in the faeces had been markedly decreased.

Determinations of the sulphaguanidine levels in the blood of the experimental animals of Groups 2 and 4 showed that a relatively constant level was

maintained under the conditions of chronic dosage, until severe renal damage had occurred. At this point the sulphonamide level rose appreciably indicating that the kidneys had lost their ability to excrete the drug and that it was piling up in the blood. That this was so was indicated by examination of the blood of the three animals in Group 4 that died before the end of the experiment. At death the free sulphaguanidine level in the blood of these animals was more than 10 times as high as the level in the living animals of the same group. At the same time the level of the conjugated sulphonamide rose four to nine times as high as the others indicating that the liver, which is presumably the site of acetylation of the drug, still possessed a high ability to conjugate the drug even at death due to toxic concentrations of the agent.

Examinations of the urine of those animals that had been kept periodically in metabolism cages indicated that the rat is able to acetylate sulphaguanidine. Both free and combined sulphaguanidine were isolated from the urines of these animals and purified by recrystallization. The melting points of the anhydrous compounds were determined and were found to be: free sulphaguanidine, 189° C.; conjugated sulphaguanidine, 265° to 268° C. These agree well with the values reported by Marshall *et al.* (8) for synthetic sulphaguanidine and acetylsulphaguanidine, respectively. The specific morphology of the urinary crystals agreed with that described by Lehr and Antopol (7) for sulphaguanidine. Moreover, hydrolysis at 100° C. of a sample of conjugated sulphaguanidine with 6*N* hydrochloric acid for 30 min. gave very closely the calculated amount of the free sulphonamide to be expected from the acetyl derivative.

It is interesting to observe that nearly all of the calculi were mixed stones containing a high proportion of phosphates. In those rats from which it was possible to obtain a urine specimen directly from the bladder at the termination of the experiment, haematuria was always observed when calculi were present. Furthermore, many epithelial and red blood cells were visible under microscopic examination of the urine. In two rats of Group 2 and four rats of Group 4 not showing calculi, epithelial and red blood cells were found in the urine as evidence of renal disturbance. The microscopic findings on the spleen, liver, and kidney are in agreement with the results on other sulphonamides (4).

Vascularization of the cornea in experimental animals is an early symptom of ariboflavinosis only when it occurs under controlled conditions. The writer has shown, and will report later, that vascularization can be produced in an animal receiving and utilizing an adequate intake of riboflavin, and in the absence of other symptoms of riboflavin deficiency, the aetiological factor in such an instance is always a trauma of chemical, physical, or physiological nature. Only 50% of the animals on the riboflavin deficient ration developed a genuine corneal vascularization, whereas of those on the riboflavin deficient ration plus sulphaguanidine, 100% developed the lesion. No empty vessels were observed in the corneas of this group and it is believed that all of them were active, blood filled vessels (13).

In consideration also of the other symptoms of riboflavin deficiency in the rat such as dermatitis, denudation, and the secretion of bloody tears, we may conclude that sulphaguanidine aggravates a chronic riboflavin deficiency presumably by depriving the organism of the source of riboflavin ordinarily present by synthesis from intestinal bacteria. The ocular and dermatological lesions are accentuated. Conversely, riboflavin deficiency seems to aggravate the severity of renal damage due to chronic sulphaguanidine dosage.

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A COMPARISON OF THE EFFICACY OF DIFFERENT INFUSION MEDIA IN SHOCK[1]

By Jean I. Hamilton[2], W. S. Hoar[3], and R. E. Haist[4]

Abstract

A procedure is described for comparing the effectiveness of different infusion media in the prevention of fatal shock. Using this procedure it was found that each of the solutions increased the chances for survival if given in adequate amounts early in shock. While 97% of the animals died without treatment, only 8% died after receiving a transfusion of plasma. Saline gave about one-third as many survivals as plasma, and the solutions of isinglass and polyvinyl alcohol were intermediate in their effectiveness.

A reliable comparison of the efficacy of different infusion media in shock is dependent upon the development of standard test procedures. Tests concerning the physical characteristics of the solutions are valuable as checks on the uniformity of different samples, but they do not give dependable information concerning the effectiveness of the material in the animal. The fundamental tests must concern the action of the various solutions in the organism itself. Various criteria such as survival or death, prolongation of survival time or maintenance of blood pressure during the period of infusion might serve as indications of effectiveness. Fundamentally, however, promotion of survival is the criterion of prime importance in work on shock. A comparison of effectiveness can be made if, with no treatment, all or nearly all the animals die in shock, whereas with the infusion of different media the number of survivals is increased, the increase being different for the various solutions.

Using a pressure cuff technique for the production of shock (1), 97% of the animals died when not treated. After shock had become well established, the infusion of different materials gave variable and generally unsuccessful results. It was found, however, that if the infusion of a suitable material was begun early enough, before shock had developed, and was given in sufficient quantities, many of the animals recovered. Since most of the untreated animals died and most of the animals treated with plasma recovered, it seemed that this procedure might be of value in comparing the efficacy of different infusion media in the prevention of fatal shock.

Methods

Dogs anaesthetized with sodium pentobarbital were shocked by the application of blood pressure cuffs to both hind legs. The pressure in the cuffs was maintained above 230 mm. Hg for five hours (1). Just before removing the

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cuffs an intravenous infusion into the brachial vein was begun. The amount of fluid administered varied somewhat in the first few animals, but subsequently the amount given was 50 cc. per kgm. of body weight. The carotid artery was cannulated and direct blood pressure records obtained. Soon after the infusion was completed, the carotid artery was ligated, the incision in the neck was sewn up, and the dog returned to its cage. Survival for 48 hr. was used as a criterion of recovery from shock. Any arbitrary criterion such as this leaves some doubtful borderline cases, but complete recovery is not a suitable criterion since the legs of these animals often become gangrenous in three to four days and complicate the study.

Four different infusion media were tested. These were saline, plasma, isinglass, and polyvinyl alcohol. Twelve dogs were tested with each solution.

The saline was given as a 0.85% solution. The plasma was fresh dog plasma. In four cases citrate was the anticoagulant and in the remainder heparin was used. No differences were noted when the different anticoagulants were used. The isinglass was a "Simple Process" solution prepared by the Connaught Laboratories according to the method of Dr. N. B. Taylor. It is a 6% solution of isinglass in physiological saline. Polyvinyl alcohol (*RII* 623) was given as a 4% solution in physiological saline*. It was prepared according to the method of Roome (5).

Results

Details of these experiments are given in Table I. When 50 cc. per kgm. of body weight of different materials were infused, the following results were obtained (Table II): saline, 33% survival; isinglass, 50%; polyvinyl alcohol, 58%; plasma, 92%. In a previous series of untreated shocked dogs, only 1 of 34 (less than 3%) survived for 48 hr. if no treatment was given. (The average survival time for untreated animals was three hours, 20 min.). Thus any of the infusions increased the chances of recovery but saline was the least effective. Plasma gave a very good incidence of survival. The other colloidal solutions used were intermediate in their effectiveness.

It is interesting to compare the ability of the different media to maintain the arterial blood pressure (Table I). In untreated dogs the blood pressure usually falls quickly after removal of the cuffs. In the infused animals the isinglass and polyvinyl alcohol were most effective in maintaining blood pressure, and saline least effective. The average reduction in blood pressure during the infusion was 35 mm. mercury with polyvinyl alcohol, 36 mm. with isinglass, 51 mm. with plasma, and 67 mm. with saline.

The plasma, although very effective in promoting survival, was somewhat less effective in maintaining blood pressure during the infusion period than was isinglass or polyvinyl alcohol. Hence there appears to be no necessary correlation between the ability of an infusion medium to bring about recovery from shock and its effectiveness in maintaining the arterial blood pressure.

* *Inclusion of this material was made at the suggestion of Dr. N. Roome.*

TABLE I

RESULTS OF INFUSION OF VARIOUS MEDIA IN SHOCKED DOGS

Weight, kgm.	Infusion material	Amount infused, cc.	Time for infusion, min.	Blood pressure, mm. Hg.		Survival
				Before cuffs off	At time infusion stopped	
7.0	Saline (0.85%)	300	35	160	142	3 hr.
22.4	Saline (0.85%)	900	90	160	120	60 hr.
7.5	Saline (0 85%)	300	35	172	80	30 hr.
7.4	Saline (0.85%)	500	60	224	112	Less than 12 hr.
15.7	Saline (0.85%)	780	60	200	154	19 hr.
18.3	Saline (0.85%)	900	55	135	54	Less than 15 hr.
21.3	Saline (0.85%)	1000	100	150	96	Greater than 48 hr.
17.0	Saline (0.85%)	850	90	160	84	Survived
12.3	Saline (0.85%)	610	60	150	102	Less than 15 hr.
11 4	Saline (0 85%)	570	50	198	142	Less than 15 hr.
15 8	Saline (0 85%)	790	85	168	88	3 days
14.2	Saline (0.85%)	715	80	175	70	Less than 15 hr.
12 2	Citrated plasma	500	50	134	110	Survived
8.8	Citrated plasma	415	60	168	100	Survived
8.0	Citrated plasma	400	35	166	60	Survived
14.3	Citrated plasma	550	60	154	110	Survived
8 4	Heparinized plasma	320	35	186	152	48 hr.
9.6	Heparinized plasma	480	60	160	104	Survived
6.3	Heparinized plasma	300	30	165	118	Survived
5 0	Heparinized plasma	250	25	192	158	Less than 14 hr.
7 0	Heparinized plasma	350	60	157	102	Survived
7.0	Heparinized plasma	350	45	164	104	Survived
10.6	Heparinized plasma	530	65	206	154	Survived
8 2	Heparinized plasma	410	40	162	136	Survived
17 5	Isinglass, 6% in physiol. saline	900	90	160	110	Survived
14.6	Isinglass, 6% in physiol. saline	750	70	168	108	Less than 15 hr.
11 0	Isinglass, 6% in physiol. saline	500	70	154	112	Less than 15 hr.
12 6	Isinglass, 6% in physiol. saline	650	75	200	156	Less than 15 hr.
6 8	Isinglass, 6% in physiol saline	340	45	172	138	Less than 15 hr.
11 4	Isinglass, 6% in physiol. saline	570	50	148	108	Less than 15 hr.
9 8	Isinglass, 6% in physiol. saline	500	80	144	116	Survived
8 1	Isinglass, 6% in physiol. saline	405	75	178	130	Survived
12 1	Isinglass, 6% in physiol. saline	605	85	160	126	Survived
10 3	Isinglass, 6% in physiol. saline	515	70	172	175	Survived
14 1	Isinglass, 6% in physiol. saline	705	90	194	164	Survived
13.6	Isinglass, 6% in physiol. saline	680	70	162	138	Less than 15 hr.
9.0	Polyvinyl alcohol, 4% in physiol. saline	500	120	174	132	46 hr.
10.5	Polyvinyl alcohol, 4% in physiol. saline	500	85	178	113	Survived

TABLE I—*Concluded*

RESULTS OF INFUSION OF VARIOUS MEDIA IN SHOCKED DOGS—*Concluded*

Weight, kgm.	Infusion material	Amount infused, cc.	Time for infusion, min.	Blood pressure, mm. Hg		Survival
				Before cuffs off	At time infusion stopped	
9.2	Polyvinyl alcohol, 4% in physiol. saline	500	120	185	158	Survived
22.4	Polyvinyl alcohol, 4% in physiol saline	900	120	150	112	Survived
19 3	Polyvinyl alcohol, 4% in physiol. saline	800	115	150	86	12 hr.
13.8	Polyvinyl alcohol, 4% in physiol. saline	650	100	162	152	6 days
9.8	Polyvinyl alcohol, 4% in physiol. saline	490	80	183	152	Survived
9.4	Polyvinyl alcohol, 4% in physiol. saline	480	70	164	154	30 hr.
8.8	Polyvinyl alcohol, 4% in physiol. saline	440	70	158	130	30-48 hr.
11.9	Polyvinyl alcohol, 4% in physiol. saline	580	70	180	174	4 days
9.7	Polyvinyl alcohol, 4% in physiol. saline	485	65	168	110	Survived
18.2	Polyvinyl alcohol, 4% in physiol. saline	910	110	150	120	30 hr.

TABLE II

SUMMARY OF RESULTS WITH DIFFERENT INFUSION MEDIA

Infusion material	Number of dogs	Number surviving 48 hr. or longer	Percentage surviving 48 hr. or longer
Physiological saline	12	4	33
Dog plasma	12	11	92
Isinglass, 6% in physiological saline	12	6	50
Polyvinyl alcohol, 4% in physiological saline	12	7	58

Recovery of the animals would thus seem to be a better criterion of the effectiveness of therapy than the maintenance of arterial blood pressure during the infusion period.

Discussion

A comparison of blood substitutes in an otherwise fatal haemorrhage was studied in a large series of dogs by Ivy and his associates (2). They found that heparinized plasma was very effective, giving 94% recovery, but that saline gave only 42% recovery and gelatine 60%. They emphasized that these results were only applicable in cases of massive haemorrhage where no

haemoconcentration was found. It is interesting that our results for a type of pressure shock in which little or no whole blood is lost and the haemoconcentration is great, are very similar to those of Ivy.

Taylor and Moorhouse (7) obtained complete recovery in 19 of 25 dogs given isinglass after a severe haemorrhage, but neither isinglass nor whole blood was effective in traumatic shock when the blood pressure had fallen to 50 mm. Hg. Mylon and his associates (4) found that citrated plasma was much more effective than saline in tourniquet shock in dogs; 78% recovered with citrated plasma, 10% with saline. Scott, Worth, and Robbins (6), however, obtained 50% recovery from shock produced by venous occlusion with infusion of 25 cc. of saline, pectin, plasma, gelatine, or polyvinyl alcohol per kgm. of body weight. Locke (3) gave infusions to shocked rats and obtained 65% survival with polyvinyl alcohol, 40% with saline and only 25% with plasma and with isinglass.

These reports indicate some of the differences in the results obtained by various groups in treating shock. While some of these variations are probably due to differences in the species studied and in the methods of producing shock, some of the variations result from differences in the times at which the infusions were started and differences in the amounts given. It seems desirable to have a testing procedure in which practically all animals die if they are not treated, but in which suitable treatment will keep a very large proportion alive. If even the best treatment available will save only 50% or less of the animals, then the test seems too severe. The method presented in this paper meets these requirements, since only 3% of the animals lived without treatment, whereas 92% lived following the infusion of plasma. A good comparison of the effectiveness of other materials with plasma can be made.

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THE EFFECT OF ACUTE STARVATION ON THE BODY ORGANS OF THE ADULT WHITE RAT, WITH ESPECIAL REFERENCE TO THE ADRENAL GLANDS[1]

BY A. T. CAMERON[2] AND J. CARMICHAEL[3]

Abstract

When rats are starved by withholding all food, though giving water, they develop, two or three days before death, a typical behaviour syndrome, in which at first there is increased activity, and subsequently, in old animals, marked immobility. At this pre-mortal stage in old animals, or the corresponding stage in younger rats, they have lost 30% or more of their body weight, and if they are killed the adrenals are found to be enlarged and discoloured to a dark grey or dark dirty grey. These enlarged glands contain an increased amount of water though their solid content remains practically unaltered, the change representing an increase in the water content of the cells (possibly confined to the cortex). The glomerular zone is diminished in thickness, and the reticular zone also shows abnormality. Animals killed before they have lost 30% of their body weight still have normal adrenals (normal in size, weight, water content, and colour). *The enlargement produced in the pre-mortal stage of inanition is a pathological process and is not a hypertrophy.* Data in the literature suggest that certain types of adrenal enlargement such as that produced by deficiency of the B-complex vitamins may be similar in nature. The kidneys, heart, and gonads of acutely starved rats tend to lose weight more slowly than the whole body; the liver and spleen more rapidly; and the thyroid at about the same rate.

Introduction

This paper embodies the results of an investigation that developed from a comparison of the relative effects of the feeding of oestrogens and of chronic starvation, whose results will be published separately. In agreement with previous reports (14, 22) we were unable to demonstrate enlargement of the adrenals in chronic starvation (5) so that the acute starvation experiments were directed particularly to determining the occurrence or otherwise of such enlargement.

Jackson (15) in 1919 found some evidence of degeneration of the fascicular zone of the adrenal cortex and of the medulla in acutely starved rats. He made no mention of enlargement. Enlargement of the adrenal glands in pigeons as result of starvation was reported by McCarrison (16) and confirmed by Vincent and Hollenberg (29) who also found it to occur in dogs and rats. It has been attributed to deprivation of B-complex vitamins during starvation (3), while the enlargement produced by a diet deficient in these vitamins has

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been attributed to resulting loss of appetite and consequent partial starvation (6), although, as mentioned above, chronic starvation does not produce adrenal enlargement.

When the earlier reports of such enlargement were made (16, 29) the function of the adrenal cortex was not known, and adrenal function was thought of in terms of adrenine content. Evidence was adduced that the adrenine content of such enlarged glands was greater than normal, so that both McCarrison and Vincent considered the enlargement to be a hypertrophy. Later evidence concerning the adrenine content of such glands is conflicting (3, 19, 20, 27, 30).

In 1943, Tepperman, Engel, and Long (27), in a critical and well-documented review of the literature dealing with hypertrophy of the adrenal cortex, found that most investigators agreed that complete inanition produces adrenal hypertrophy. Goldzieher (11) in a still later review holds the same view.

In most acute starvation experiments water has not been withheld.

Experimental Part

In all experiments an equal number of control rats were kept in the same environmental conditions, and fed the usual diet of unlimited bread and milk and table scraps during the experimental period. The starved animals were given unlimited water. All animals were isolated in separate cages.

The earlier experiments were carried out with fairly old female rats. (In an initial measurement of the survival period, that of six animals varied from six to nine days; they lost from 31 to 39% of body weight before death.) It was found that animals killed after five days' starvation seldom showed adrenal enlargement, and the period was gradually extended. Careful observation indicated a fairly regular pattern of behaviour in these elderly animals. During the first few days nothing unusual was observable. About 48 hr. before death would occur the starved rats became more active than the controls, and the increased activity persisted at least 24 hr. and sometimes 36 hr. and more. Subsequently the animals became inactive and were usually curled up in a ball, scarcely responding at all to such a stimulus as tapping the cage. At this period they had always lost at least 30% of body weight. Those animals allowed to live longer died during the following night or were moribund next morning. If they were killed (by chloroforming) during the day on which the immobility was first observed or subsequently, before actual death from starvation took place, the adrenals were almost always found to be definitely enlarged, and of a dark grey (or dark dirty grey) colour. Such animals did not struggle while going under the anaesthetic, but breathed slowly and deeply for a short while and then suddenly keeled over.

Old male rats behaved similarly. In younger animals the onset of immobility was less noticeable or did not occur. In the late stage with these younger animals, when they had lost well over 30% of their weight, the eyes remained partially closed, and the stance of the animals was continuously "compressed". Killed at this stage these animals also almost always showed enlarged and discoloured adrenals.

In all, 12 experiments were carried out with 70 rats, half of which were starved. Of the latter, three died during the night and were not examined, two were examined just after death had occurred, and the remainder were killed with chloroform. The controls were killed at about the same time. The adrenals in eight controls and seven starved animals, and the thyroids in five controls and five starved animals were examined histologically. The method of dissection, etc., employed has been described elsewhere (4).

The general results are most easily recorded by separating the starved animals into two groups, (i) animals in which the loss of body weight was less than 30%, and (ii) those in which it was 30% or more. Variations in the organ weights of different animals, and indeed in the individual percentage organ weights, were considerable, both in controls and starved animals, and averages probably give a sufficiently accurate idea of the general results.

In the older rats the change in weight of the controls during the brief experimental period was so small (usually within $\pm 2\%$) that their final weights could properly be used to calculate the percentage organ weights, but the initial body weights of the starved animals had to be used. In younger and still growing animals, conclusions were drawn by contrasting percentage organ weights based on initial with those based on final body weights in the manner described later.

Rate of Loss of Body Weight

For 17 fairly old females (initially 186 to 259 gm.) the rate of loss of body weight averaged for the first few days about 8 gm. daily (extremes 4 to 10 gm.). There was no definite relation between initial weight and weight loss. For six old males (178 to 322 gm.) the average loss was about 10 gm. (extremes 8 to 12 gm.). For seven younger males (112 to 134 gm.) the average daily loss was about 7 gm. (extremes 6.5 to 8 gm.). There was a general tendency to a slightly more rapid fall of weight in the last day or two.

Loss of Weight of Certain Body Organs in Older Animals

The starved animals showed general loss of body fat. The amount was not determined. The average results for the body organs examined are shown in Table I.

TABLE I

GAIN OR LOSS OF CERTAIN BODY ORGANS OF OLD RATS PRODUCED BY STARVATION

Sex	No.	Original body wt., gm.		Loss of body wt., %		Average loss or gain of organs, %							
		Extremes	Mean	Extremes	Mean	*L*	*K*	*H*	*S*	*M*	*G*	*A*	*T*
Female	9	192 to 257	214	−16 to −29	−22.5	−30	−16	−13	−23	−22	− 5	− 2	− 9
	9	186 to 259	213	−31 to −45	−36	−50	−24	−23	−52	−30	−16	+29	−30
Male	2	224 to 322	273	−24 to −26	−25	−38	−27	−21	−19	−14	− 6	− 7	+30*
	3	226 to 314	279	−30 to −34	−32	−68	−33	−30	−53	−23	−18	+20	−31

* *One thyroid was greatly enlarged for some unascertained reason. Its water content was normal.*

L, liver; K, kidneys; H, heart; S, spleen; M, right anterior tibialis muscle; G, gonads (ovaries or testes); A, adrenals; T, thyroid.

The results with female rats indicate that during the period when weight loss is less than 30% the liver loses weight relatively more rapidly than the whole body; the spleen and muscle at about the same rate; kidneys, heart, thyroid, and ovaries more slowly; while the adrenals are practically unaffected. With greater loss of body weight the spleen also loses weight relatively faster than the whole body, the others relatively less rapidly, while the adrenals gain weight.

The number of male animals was too small to stress the results obtained with them, but the general picture is the same.

TABLE II

EFFECT OF STARVATION WEIGHTS ON WEIGHTS OF BODY ORGANS OF YOUNG ADULT MALE RATS

	Rat 1 (control)	Rat 2 (control)	Rat 3 (starved)	Rat 4 (starved)
Body weight, gm.				
Initial	111	114	114 5	112
After 5 3 days	—	—	—	69 5
After 6 0 days	137	135.5	—	—
After 6 3 days	—	-	69 5	—
Gain or loss	+26	+21 5	−45 (−39%)	−42 5 (−38%)
Organ weights, gm.				
Liver	8 7	9 6	3 4	2 0
Kidneys	1 62	1 52	0 98	0 84
Heart	0 47	0 47	0 29	0 30
Spleen	0 48	0 45	0 11	0 11
Muscle*	0 26	0 24	0 17	0 14
Testes	1 82	1 56	1 33	1 35
Adrenals	0 0280	0 0302	0 0355	0 0365
Thyroid	0 0135	0 0149	0 0074	0 0070
Dry weights: adrenals	0 0088 (31%)	0 0087 (29%)	0 0091 (26%)	0 0087 (24%)
Organ weights referred to orig. body wt., %				
Liver	7.8	8 4	2 1	1 8
Kidneys	1.46	1.33	0 86	0.74
Heart	0 42	0.41	0 25	0 26
Spleen	0 40	0 40	0 09	0 09
Muscle*	0.23	0 21	0.15	0.12
Testes	1.64	1 37	1.16	1.19
Adrenals	0 0252	0 0265	0 0310	0.0320
Thyroid	0.0121	0.0131	0 0065	0 0061
Organ weights referred to final body wt., %				
Liver	6.4	7.1	4.8	2.9
Kidneys	1 18	1.11	1.27	1.21
Heart	0.34	0.34	0.41	0.42
Spleen	0.35	0.34	0.16	0.15
Muscle*	0 19	0.18	0.25	0.20
Testes	1.33	1.15	1.91	1.94
Adrenals	0.0204	0.0223	0.0511	0.0532
Thyroid	0.0098	0.0110	0.0106	0.0101

* *Right tibialis anterior muscle.*

The conservation of weight of the gonads seemed to be associated with conservation of function. About half of the relatively old female rats (both starved and control animals) showed numerous haemorrhagic follicles.

Results with Younger Animals

The protocol of a typical experiment with young rats is given in Table II. Four males of the same litter, born Dec. 2, 1944, were used, and the experiment was started on the 53rd day of age.

It seems proper to analyse results such as those in Table II in the following way:

	Compared with controls	Conclusion
Actual organ weights	Greater	Actual enlargement
Percentage organ weights referred to initial body weight	Greater	Actual enlargement
Percentage organ weights referred to initial body weight	Equal	No effect
Percentage organ weights referred to initial body weight	Less	Relatively less loss than that of whole body
Percentage organ weights referred to final body weight	Greater	Relatively less loss than that of whole body
Percentage organ weights referred to final body weight	Equal	Loss proportional to that of whole body
Percentage organ weights referred to final body weight	Less	Loss relatively greater than that of whole body (with possible actual atrophy)

Applying such analysis to the results in Table II, they indicate (*a*) atrophy of liver and spleen, (*b*) loss of weight of kidneys, heart, muscle, and testes less in degree than that of the whole body, (*c*) loss of weight of the thyroid proportional to that of the whole body, and (*d*) actual enlargement of the adrenals.

Other experiments with young adult male rats, in which body weight loss exceeded 30%, were in complete agreement. It will be seen that the results are in general agreement with those obtained with older animals of both sexes.

Effect of Starvation on the Adrenal Glands

The abnormal appearance of the adrenal glands of animals whose loss of body weight was 30% or more led to determination of their water content. This was done in all cases except when glands were required for histological examination. They were dried for 24 hr. at 102° to 105° C. Results are shown in Table III for, in order, (i) old females, (ii) old males, (iii) younger males. Data for controls precede those for starved animals. The latter are arranged in order of decreasing water content of the adrenals. Presence or absence of enlargement of the adrenals was judged by direct comparison with the adrenals of corresponding controls, and also comparison based on initial body weights. Some of the exceptions to general findings may be due to individual variations.

It is evident that when there is a loss of body weight of 30% or more, in almost every case the adrenals become enlarged, and at the same time dis-

TABLE III

EFFECT OF STARVATION ON THE ADRENAL GLANDS

Sex	No. of animals	Treatment	Duration, days	Initial body wt., gm.	Gain or loss in body wt., %	Adrenals			
						Fresh wt., gm.	Dry wt., %	Colour	Size
Female	11	Control	(5 to 11)	176 to 230	−1 to +3	0.0467 to 0.0606	30 to 33	Normal	Normal
	1	"	(8)	217	+1	0 0954	27	Dark grey	Large
	1	"	(11)	226	−1	0 0847	30	Greyish	Large
	1	"	(8)	214	+2	0 0746	32	Grey-yellow	Large
Female	1	Starved	11	245	−45	0 1762	16	Dark dirty grey	Enlarged*
	1	"	11	259	−39	0 1012	21	"	"
	1	"	9	205	−40	0 0586	22	"	?
	1	"	5	189	−32	0 0692	23	Dark grey	Enlarged**
	1	"	7	202	−32	0 0649	23	"	"
	1	"	7	186	−36	0 0569	23	"	"
	1	"	8	211	−31	0 0802	27	Almost normal	"
	7	"	5 to 8	192 to 257	−29 to −16	0 0488 to 0 0637	27.5 to 36	Normal	Normal
	1	"	5	217	−18	0.0665	33	Normal	Enlarged
Male	5	Control	(7 to 9)	224 to 325	−1 to +2½	0 0268 to 0 0376	29 to 33	Normal	Normal
	1	"	(9)	311	−1	0 0497	26	Bright yellow	Large
Male	3	Starved	6 to 9	226 to 296	−30 to −34	0 0359 to 0.0459	23 to 23	Dark grey	Enlarged
	2	"	(7, 9)	224, 322	−24, −25	0 0277, 0 0355	28.5, 29	Normal	Normal
Male	5	Control	(6 to 7)	97 to 137	+12 to +23	0.0199 to 0 0322	29 to 37	Normal	Normal
Male	1	Starved	5 2	112	−38	0 0365	24	Dark grey	Enlarged
	1	"	<6	134	−36	0 0337	25	(Dark red)***	Enlarged
	1	"	6 3	131	−34	0 0257	25	Dark grey	Normal
	1	"	6 2	114	−39	0 0355	26	Dark dirty grey	Enlarged
	1	"	6 3	129	−31	0 0278	28	"	Normal

* *Adrenals swollen like tiny balloons. When punctured after transfer to weighing vessel, a clear fluid exuded.*

** *When adrenals were punctured after transfer to weighing vessel, a blood-stained fluid exuded.*

*** *Animal died during night but was still warm and not in rigor when examined. The dark red colour of the adrenals was presumably a post-mortem change.*

coloured to a dark grey or dark dirty grey (often like dark mud), while their water content increases. (Occasionally such enlarged glands showed one or two apparently haemorrhagic spots on their surfaces.)

That the content of solid of these enlarged glands did not appreciably alter is shown in Table IV. (Final body weights were used in calculating the ratio of adrenal dry weight to total body weight for the older controls, and initial body weights for all starved animals and for the younger controls.)

Histological Examination

Sections of adrenals and thyroids were preserved in 10% commercial formol, or in Bouin's solution, and stained with haematoxylin-eosin. In preliminary experiments no striking differences were observed in the adrenals of starved

TABLE IV

EFFECT OF STARVATION ON THE SOLID CONTENT OF THE ADRENALS

Group	No. of rats	Adrenal water content, %	Adrenal solid content, %	Ratio of adrenal dry weight to body weight	
				Extremes	Mean
Control females (old)	14	67 to 73	33 to 27	0.0060 to 0 0118	0.0088
Starved females (old)	8	64 to 72½	36 to 27½	0.0072 to 0.0111	0.0084
Starved females (old)	7	73 to 84	27 to 16	0.0063 to 0.0118	0.0085
Control males (old)	6	67 to 74	33 to 26	0 0033 to 0.0043	0.0037
Starved males (old)	2	71 to 72	29 to 28	0.0031 to 0.0036	0.0033
Starved males (old)	3	77	23	0.0030 to 0.0037	0.0034
Control males (young)	5	63 to 71	37 to 29	0.0061 to 0.0087	0.0073
Starved males (young)	5	72 to 76	28 to 24	0.0048 to 0.0098	0.0073

and control animals. The cells lining the thyroid alveoli of starved animals tended to be flatter, and the alveolar colloid to stain less.

Since chemical evidence indicated a definite increase in water content in almost all the cases of adrenal enlargement, the only possible conclusion seemed to be that the average cell size was greater. It was thought that a count of nuclei in comparable areas might test this conclusion. In two preliminary experiments, using the method of counting described below, the nuclei in comparable areas were respectively 4 and 13% less in the adrenals of starved rats than in the controls. A third, more thorough experiment was then performed, and is described fully.

Six male rats of the same litter were used. Starvation of three was begun on the 48th day of age; the others served as controls. One of the starved animals died on the sixth day, during a week-end. The pertinent data for the others are given in Table V.

The adrenals were preserved in Bouin's solution (water, 30 parts; formalin, 10 parts; 2% aqueous trichloracetic acid solution, 2 parts; and picric acid to saturation), sectioned, and stained with haematoxylin–eosin. All sections were 7μ thick. As far as possible, sections were cut at right angles to the longest adrenal axes and to their lower (flatter) sides adjacent to the kidneys. Photomicrographs were made from sections from the central portions of the glands.

Except where flaws in the sections interfered, attempts were made to obtain four photomicrographs from the four opposite points of the peripheries of a section, selecting, where possible, areas with fairly flat peripheries.

In all, 23 photomicrographs were made of the four adrenals of the starved animals, of which 20 (5 × 4) were made from five sections; 29 were made from the six adrenals of the controls, of which 20 (5 × 4) were from five sections.

Where possible, square 10 × 10 cm. areas were used to count nuclei, each area bordering on the peripheral connective tissue but just excluding it. In

TABLE V

DATA CONCERNING RATS USED IN THIRD HISTOLOGICAL EXPERIMENT

	Rat 1 (starved)	Rat 2 (starved)	Rat 4 (control)	Rat 5 (control)	Rat 6 (control)
Weight, gm.					
Initial	126 5	127	121	122 5	124.5
After 7.3 days	75 5	72.5	—	—	—
After 8 days	—	—	146.5	148 5	150
Gain or loss in weight	−51 (−40%)	−54 5 (−43%)	+25.5	+26	+25.5
Right adrenal gland	0 0166	0 0186	0 0131	0 0150	0.0150
Left adrenal gland	0 0190	0 0180	0.0127	0 0169	0.0147
Total adrenal weight	0 0356	0 0366	0 0258	0 0319	0.0297
Average (normals)				(0 0291)	
Approximate weight increase (starved rats)	+22%	+26%			
Adrenal colour	Dark dirty grey	Dark dirty grey	Slightly greyish yellow	Slightly greyish yellow	Dull yellow

each square, the nuclei were counted in alternate strips, each 1 × 10 cm., running inwards from the periphery. When the edge was markedly curved, five consecutive strips in a total area of 5 × 10 cm. were counted. All such strips included the glomerular and fascicular zones and a portion of the reticular zone.

Counts in 10 consecutive strips and in five alternate strips gave an average difference (11 comparisons) of 0.35% and a maximum difference of 1 7%. Eleven similar comparisons between 10 and 5 consecutive strips gave an average difference of 1.5% (maximum 3.3%).

All "spots" that could possibly be nuclei were counted. Hence the counts were undoubtedly inaccurate and too great, but it seems reasonable to assume that the errors for starved and for control animals were of similar magnitude.

The results showed a random variation with respect to the position of the area counted (adjacent or opposite to the base of the adrenal, etc.). Moreover, counts made in several instances from more than one section of the same gland showed marked differences, although such sections were fairly close to each other (the actual distance apart was not determined). Thus typical actual figures were, per square cm.:

Rat 2 (starved), right adrenal,—

Section A, four photos: extremes 28.2–30.8; mean 29.4.

Section B, four photos: extremes 19.4–22.7; mean 21.0.

Rat 4 (control), left adrenal,—

Section A, three photos: extremes 40.5–47.3; mean 44.9.

Section A, four photos: extremes 35.0–37.8; mean 36.2.

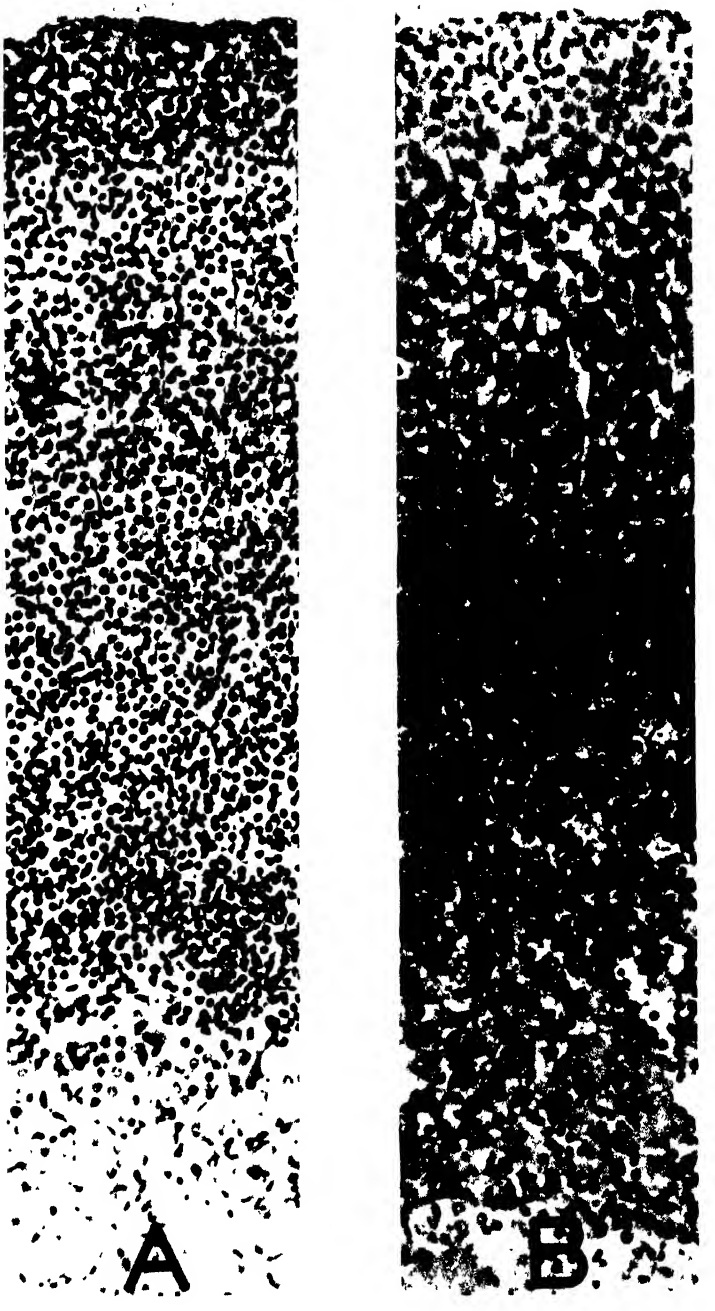

FIG. 1. *Photomicrographs of adrenal sections. ×200. A, left adrenal, Rat 4 (control). In an area 10 × 10 cm., bordering on the periphery, a nuclear count showed 47.3 nuclei per square cm. B, right adrenal, Rat 2 (starved). The corresponding count was only 22.6 nuclei per square cm.*

Hence it seemed useful to compare only the over-all averages, and the averages for the total counts of each adrenal. These (per square cm.) were as follows:

23 photos of sections of 4 adrenals of 2 starved rats averaged 30.2
29 photos of sections of 6 adrenals of 3 control rats averaged 36.4

Decrease in starved animals, 17.0%.

7 photos of left adrenal of starved rat No. 1 averaged 32 1
4 photos of right adrenal of starved rat No. 1 averaged 32.8
4 photos of left adrenal of starved rat No. 2 averaged 34 3
8 photos of right adrenal of starved rat No. 2 averaged 25 2

Mean, 31.1.

7 photos of left adrenal of control rat No. 4 averaged 39 9
4 photos of right adrenal of control rat No. 4 averaged 43 5
4 photos of left adrenal of control rat No. 5 averaged 31.4
6 photos of right adrenal of control rat No. 5 averaged 34 0
4 photos of left adrenal of control rat No. 6 averaged 35 6
4 photos of right adrenal of control rat No. 6 averaged 32 7

Mean, 36 2.

Decrease in starved animals, 14.1%.

On a volume basis the last figure indicates a difference of about 25%, while the average enlargement in the starved animals was about 24%. This close approximation can only be accidental, since only a small part of each gland was examined, and the medullas not at all.

As is to be expected counts of areas in individual photos of the two groups showed considerable overlapping. The extremes were, for starved animals, 19.4 and 37 0 per square cm., and for controls 29.3 and 47.8.

The average results are in agreement with the supposition that the increase in water content (and lack of increase in solid content) of adrenals of starved rats is accounted for by enlargement of the cells.

Fig. 1 shows photomicrographs of two extreme cases. These indicate that there is a considerable narrowing of the glomerular zone in the starved rats; such decrease was generally present in some degree, as indicated both by inspection of the series of photographs and camera lucida drawings of actual sections. The reticular zone also tends to be abnormal, with frequent spaces empty of nuclei, and presumably indicating a degenerative process. Fig. 1 also suggests that a large proportion of the nuclei in the adrenals of the starved animals are themselves enlarged, sharing in the increased water content.

Study of camera lucida drawings of the sections suggested that the medullas were not enlarged; there were, however, considerable variations of the areas in different sections, and this conclusion cannot be stressed.

Discussion of Results

The effect of starvation on the body organs of the white rat (excluding the adrenals), as reported in this paper, is in fairly good agreement with the results of Jackson (14) and of Mulinos and Pomerantz (21). The latter investigators withheld water as well as food, and apparently this added deprivation does not materially affect the results.

Vincent and Hollenberg (30) claimed that inanition produces enlargement of the thyroid in the rat. (Some of their published values are at least 10 times too great, apparently through misplacement of a decimal point in printing.) No later observer seems to have confirmed this claim, nor do our findings support it.

The increase in activity for two or three days prior to death in the acutely starved rat accords with observations of Hoskins (13) and of Hitchcock (12).

The fairly constant pattern of behaviour, associated with a fairly definite degree of weight loss, enables animals to be killed at a time when in almost all cases the adrenals are definitely enlarged. To ensure this, the rats must have been unduly active for at least 24 hr., old animals must have reached the subsequent quiescent stage, and body weights must have decreased by 30% or more.

Rats killed at any period when weight loss is less than 30% have normal adrenals.

These findings are in agreement with, and are somewhat more precise than those of Mulinos and Pomerantz for rats (22), and Oleson and Bloor (24) for guinea pigs. The former stated: "At the end of the first week of starvation the adrenal glands are usually heavier, although they may be normal and at times reduced in weight. As the period of starvation lengthens and the body weight loss becomes more severe, the adrenal glands increase in weight. When the starvation was allowed to continue until the rats became moribund, the adrenal glands were invariably enlarged." However, they considered that the enlargement represented true physiological growth (23). Oleson and Bloor reported that the weights of guinea pig adrenals are not changed by a seven day fast, although they are increased in animals dying in hunger.

Our results show that the water content of normal adrenals varied slightly, but was never greater than 73% in old females, 74% in old males, and 71% in younger males. In starved rats enlarged adrenals almost invariably showed a greater water content, usually about 76 to 77%, the maximum found being 84%. Marrian reported similar findings in pigeons (18).

The ratio between the solid content of such adrenals and the original body weight did not appreciably change from normal, indicating that there had been no real loss of solid, nor any gain. This is in agreement with the findings of Addis, Poo, and Lew (1) that rat adrenals after a seven day fast showed no loss or gain in protein content. Data in the literature concerning adrenal lipides in starvation give no definite clue to any over-all lipide change (24, 28, 31), though there is possibly some depletion (25).

Nuclear counts of specified areas in sections of adrenals enlarged from starvation showed fewer nuclei than normal, apparently an indication of fewer and larger cells per unit area. The nuclei also tended to be larger. Such sections showed a narrower glomerular zone, and some evidence of degeneration of the reticular zone (cf. 15).

The change in water content, with unaffected solid content, indicates that adrenal enlargement resulting from acute starvation is due to an increased

uptake of fluid by the cells. *It is not a hypertrophy, but is perhaps in the nature of a hydropic degeneration.*

Perhaps bearing on this point are the observations of Mulinos and Pomerantz (22) that peritoneal tissues adjacent to the adrenals often become oedematous in such animals, and those of Barlow and Whitehead (2) that in such acute starvation there is evidence of a general hydraemic plethora.

(An opposite condition, increase of the solid content of the adrenals of rabbits chronically poisoned by ammonia, has been reported by Fazekas (8).)

Edelmann (7) has demonstrated that a significant enlargement of the adrenals of rats subjected to anoxia through decreased pressure is due to a hyperaemia.

The normal rat adrenal, as seen in our experiments, is yellow, dull yellow, or greyish yellow, sometimes with a faintly reddish tinge. In one or two cases (cf. Table III) in apparently normal animals the adrenals were dark grey or greyish and unduly large; they possibly were not normal. Glands enlarged through starvation were almost invariably dark grey, or what is best described as dark dirty grey.

This change in colour, accompanied by increase in water content of the cells, indicates that in the final stage of starvation (but only then) a pathological process sets in in these glands. In extreme cases it probably develops to a marked degeneration, as indicated by the first two asterisked cases in Table III, in which, when the glands were punctured, free fluid exuded. Unfortunately such extreme cases cannot be produced at will experimentally.

Whether the onset of this pathological condition can be related to the earlier increased activity cannot be stated.

It seems probable that whenever experimental treatment results in marked discoloration of the adrenals, this indicates presence of a pathological process. Mulinos and Pomerantz (22) stated that those of their starved rats "were large and red with a cortex that was friable and congested with blood." It is to be remembered that water was withheld from their animals.

Colour changes resulting from other treatment have been recorded. Thus McCarrison (17) noted that pigeons on a sole diet of milled and autoclaved rice developed enlarged yellow-red or more commonly red-brown adrenals (cf. also Findlay (9)). The condition of B-complex deficiency in pigeons shows, indeed, a very close parallel to that in starved rats. Marrian (18) demonstrated an increased water content of the adrenals, and McCarrison (17) stated that equal areas of adrenal sections showed fewer cell nuclei than normal. Hence in this condition also something in the nature of a hydropic degeneration may be postulated.

Selye (26) found that in rats poisoned with formaldehyde or some other drug or subjected to severe exertion or to cold the adrenals became enlarged and dark brown. Glock (10) very recently reported that in the terminal stages of fatal poisoning by thiourea or thiouracil rat adrenals were frequently enlarged and dark reddish brown in colour. We have ourselves observed a similar change following chronic dosage with oestradiol (5).

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DETERMINATION OF THE EFFECT OF IRRADIATION BY X-RAY ON *p*-AMINOBENZOIC ACID, USING *CLOSTRIDIUM ACETOBUTYLICUM* FOR ASSAY[1]

By Ivan C. C. Tchaperoff[2]

Abstract

A study has been made on the effect of X-ray irradiation by producing inactivation of *p*-aminobenzoic acid as determined by assay with *C. acetobutylicum*. It has been shown that *p*-aminobenzoic acid in concentration of 1×10^{-4} μgm. per cc. is inactivated by an X-ray dose as small as 50 *r* at 190 kv. The significance of the destruction of Paba in connection with the treatment of infective lesions is discussed.

The importance of *p*-aminobenzoic acid (Paba) as a bacterial growth factor is to-day well established. According to the criteria enunciated by Fildes (2) Paba is a substance probably concerned in the metabolism of most bacteria and one that may possibly play a role in the cellular metabolism in general. The extraordinary biological activity of this compound as a bacterial growth factor suggests that it may be chemically related to a co-enzyme. Park and Wood (7) and Rubbo and others (8) have proved conclusively that Paba functions as a bacterial growth factor for *Clostridium acetobutylicum*, which is unable to produce this metabolite from the media in which it grows.

In this report it is demonstrated that the destruction of Paba by X-ray irradiation can be used as a means of producing bacteriostasis in a way similar to the mechanism by which sulphonamides are believed to act.

Tchaperoff (9) has reported that sulphonamides and Paba in concentrations of the order of 10 mgm. per 100 cc. are broken down by very heavy doses of irradiation. The breakdown was measured by Marshall's test. As described by Marshall (5) this test cannot be used for measuring solutions lower than 0.5 mgm. using the Klett Biological colorimeter. It was therefore decided to use the method described by Park and Wood (7) to investigate the breakdown following irradiation of Paba in concentrations of 1×10^{-4} μgm. per cc.

Materials and Methods

Clostridium acetobutylicum 862, obtained from the American Type Culture Collection, grows well anaerobically on an infusion of corn meal. The corn meal infusion tubes are autoclaved for one hour at 15 lb. pressure before inoculation. After inoculation the tubes are evacuated and sealed by a blow torch. Daily transfers are made to fresh corn meal tubes so that very vigorous growth is obtained in 24 hr.

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Contribution from the Associate Committee on Army Medical Research, National Research Council of Canada.

[2] *Major, R.C.A.M.C.*

The basal medium used is the same as that described in papers by Park and Wood (7) and Oxford *et al.* (6). It consists of: glucose, 20.6 gm.; asparagine, 1.0 gm.; sodium chloride, 0.01 gm.; ferrous sulphate heptahydrate, 0.01 gm.; manganese chloride tetrahydrate, 0.01 gm.; reduced iron, 3 to 5 mgm.; distilled water, 1000 cc. The glucose is made up separately into solution and added to the rest of the basal medium just before use. Both solutions are kept as stock solutions at four times the above concentration. They are sterilized by autoclaving for 15 min. at 15 lb. pressure.

The *C. acetobutylicum* is transferred from corn meal tubes to the basal medium enriched with 1.0% peptone. On this medium, growth takes place vigorously in 36 to 48 hr. in test-tubes that are evacuated and sealed as for the corn meal tubes. Any transfer of corn meal is avoided by three to four serial transfers on the peptone medium. All incubations are at 37° C. From a vigorous growth on the peptone enriched medium 30 cc. of the culture is centrifuged, the supernatant is discarded, and the residue suspended in 20 cc. of basal medium. As described by Park and Wood, this is repeated three times to get rid of any peptone that may be carried over. Final suspension is made up in 20 cc. of basal medium for inoculation.

Crystalline biotin supplied by Merck and Co. is made up to 1.5×10^{-4} μgm. per cc. in distilled water. Park and Wood have shown that biotin is necessary for growth on this basal medium in concentration of at least 1.5×10^{-5} μgm. per cc.

Chemically pure Paba supplied by Eastman Kodak is made up to 1×10^{-3} μgm. per cc. in distilled water. Park and Wood have shown that Paba is extremely active as a growth-factor in concentrations of 1×10^{-2} to 1×10^{-5} μgm. per cc. Great care has to be taken in cleaning all glassware as at first contradictory results, which were due to Paba contamination, were obtained.

Paba in a concentration of 1×10^{-3} μgm. per cc. and biotin 1.5×10^{-4} μgm. per cc. were irradiated separately in sealed glass test-tubes with the following factors: −190 kv., 20 ma., 4 mm. tin filter, 40 F.S.D.* Test-tubes in pairs containing Paba and biotin solutions were given 50, 100, 150, 200, 400, 800, and 1200 *r* respectively. As will be seen later the Paba is destroyed.

The method of using a McIntosh and Fildes anaerobic jar was found unsatisfactory for growth over 48 hr. Contaminations appear to take place from the 'moist oats' method described by Park and Wood. Modified Turnberg tubes proved a very satisfactory solution of the problem. They are made to fit exactly into the Evelyn photoelectric colorimeter, which is used for measuring the opacity of the growth. The tubes have an added constriction made one inch below the tube neck. This space was packed with a cotton wool plug to prevent contamination of the medium. The tubes are of sufficient

* *Focus Skin Distance.*

length so that 20 cc. of medium fills them only one-third. A second glass trap packed with cotton wool is attached to the side arm outlet of the tube. After the tubes are filled with the medium, sterilized, and inoculated, they are evacuated by the vacuum pump and the vacuum renewed every 24 hr. When it is desired to measure the degree of opacity of the growth, the tubes are placed directly in the colorimeter. This obviates the necessity of removing at least 5 cc. samples each day for determination of growth and lessens the chances of contamination.

Each tube contained: 5 cc. of the stock solutions of glucose in quadruple strength; 5 cc. of the stock solution of salts in quadruple strength; 5 cc. of the biotin solution to give a final concentration of 1.5×10^{-5} μgm. per cc.; and 5 cc. of the Paba solution to give a final concentration of 1×10^{-4} μgm. per cc., thus making up 20 cc. in each tube of the required concentrations. Table I shows the contents of the tubes and the amount of radiation in "*r*" given to the biotin and Paba solutions before final dilution. The tubes were inoculated with 0.5 cc. of the *C. acetobutylicum* inoculum, which had been suspended, as described, in 20 cc. of basal medium free from peptone, biotin, or Paba. It was found necessary to use the inoculum when it was growing very vigorously otherwise it died out in the tube. Park and Wood also found it necessary to use as large an inoculum as 0.5 cc.

The tubes were incubated at 37° C. and the degree of opacity measured each day by placing the tubes directly in the Evelyn photoelectric colorimeter.

TABLE I

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

Addition to basal medium	Radiation in *r*	Tube No.	Time in days								
			0	1	2	3	4	5	6	7	8
Radiated Paba + radiated biotin	50	1	93	93	92	92	92	92	92	82	82
	50	2	93	93	92	92	92	92	92	82	82
	100	3	95	95	92	92	92	92	91	73	71
	100	4	94	94	90	90	90	90	90	70	70
	150	5	91	91	89	89	89	89	89	70	79
	150	6	93	93	90	90	89	84	81	71	70
	200	7	93	93	90	91	91	91	91	82	80
	200	8	92	92	91	91	91	91	91	82	80
	400	9	90	90	90	89	89	87	89	75	78
	400	10	91	91	90	90	90	91	91	78	80
	800	11	90	90	90	90	88	80	78	67	65
	800	12	91	91	89	89	89	89	89	82	82
	1200	13	93	93	90	89	89	89	89	82	80
Normal Paba + normal biotin	Nil	14	89	89	89	37	37	42	35	35	36
	Nil	15	91	91	83	43	42	42	45	42	45
No Paba and no biotin	Nil	16	91	91	89	89	89	82	77	70	76
	Nil	17	90	90	89	89	89	88	72	74	72

NOTE: *Opacity measured by Evelyn photoelectric colorimeter (base line 60).*

Results

The degree of opacity produced by bacterial growth in each tube is shown in Table I; as the growth increases the readings decrease. The effect of radiated Paba and radiated biotin had been added and demonstrates the destruction or inactivation of the Paba by irradiation. Table II shows no

TABLE II

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

	Time in hours	
	48	64
Basal medium only	0	0
Basal medium + Paba + biotin	0	+++
Basal medium + radiated Paba + radiated biotin	0	0
Basal medium + radiated Paba + biotin	0	0
Basal medium + Paba + radiated biotin	0	+++

NOTE *No growth indicated by 0; marked growth indicated by* +++.

evidence of destruction of the biotin and the whole change is due to the inactivation of the Paba. As all the tubes containing radiated Paba show such a close comparison the results are averaged in Table III from which Fig. 1 is constructed. The growth on basal medium with Paba and biotin

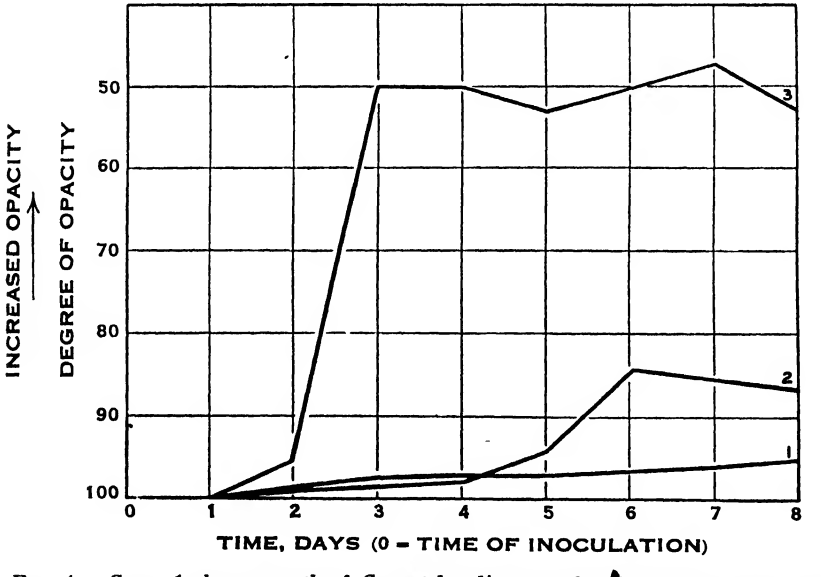

FIG. 1. *Curve 1 shows growth of C. acetobutylicum on basal medium with addition of radiated Paba and radiated biotin. Curve 2 shows growth of C. acetobutylicum on basal medium without Paba or biotin. Curve 3 shows growth of C. acetobutylicum on basal medium with addition of Paba and biotin.*

TABLE III

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

	Time in days						
	0	1	2	3	4	5	6
Average figure of readings from tubes containing *radiated biotin* and *radiated Paba* at 195 kv., 50–1200 *r*.	92.2	90 4	90 4	90.3	90	89	88 8
Average figure of readings from tubes containing *unradiated biotin* and *Paba*	90	90	86	40	39.9	42	40
Average figure readings from tubes containing only basal medium without *biotin* or *Paba*	90 5	90 5	89	89	89	85	74.5

NOTE: *Opacity of growth measured by Evelyn photoelectric colorimeter (base line 60).*

(Curve 3, Fig. 1) reaches nearly the maximum at the third day, whereas little or no growth occurs on the basal medium with radiated Paba and radiated biotin (Curve 1, Fig. 1). As control, Curve 2 (Fig. 1) shows the growth on basal media without the addition of Paba or biotin.

Discussion

The results demonstrate that as little as 50 *r* can inactivate Paba as a growth factor when radiated in a concentration of 1×10^{-3} μgm. per cc. and diluted to 1×10^{-4} μgm. per cc. in the basal medium. This is well within the known concentration for the demonstration of the biological activity of Paba by *C. acetobutylicum*. It is important to radiate the Paba as nearly as possible at the concentration in which it will occur in the medium, if changes are to be produced by small doses of irradiation. The fact is brought out by Dale (1) in discussing the effects of X-rays on enzymes. Dale irradiated carboxypeptidase and showed that X-rays have a qualitatively uniform effect on enzymes over the whole range from very small doses to doses 1000 times as large—(400 to 400,000 *r*). Fricke and Hart believe that the changes are produced by "X-ray activated water" and that it is in the nature of an oxidation process. Hydrogen peroxide is hypothetically believed to be formed but exists in the solution only momentarily.

The method used by Park and Wood, which has been followed in this report, is only semi-quantitative for *p*-amino acid. It is desirable to repeat the experiments using the more quantitative method of Lampen and Peterson (3) with an assay range 0.3 to 1.5 mμgm. of Paba, the error being 10% or that of Lewis (4) with a sensitivity range of 0.15 to 0.5 mμgm. of Paba. The latter uses an aerobic culture method and the acid produced in the medium is titrated with 0.1*N* sodium hydroxide using bromothymol blue as an indicator.

Park and Wood give the minimum concentration of Paba in the basal medium at which *C. acetobutylicum* will grow as 1×10^{-5} μgm. per cc.; our

experiments indicate that the irradiation reduces the Paba from 1×10^{-4} to below 1×10^{-5} μgm. per cc.

It is believed that the demonstration of the destruction by Paba by irradiation indicates the part played by X-rays in the treatment of infection. X-ray treatment makes it possible to treat infected areas intensely since the irradiation in the doses used in treatment has no systemic effect. Because it has been demonstrated that Paba can be destroyed by irradiation, it is suggested that X-ray treatment is preferable to sulphonamides in infections that are sulphonamide resistant and that produce large amounts of Paba either by synthesis or by freeing it from the tissues.

It remains to be demonstrated whether irradiation can reduce the Paba concentration in suitable basic media faster than the organism can produce it and thus can produce bacteriostasis. Such a demonstration would offer a reason for the use of X-ray therapy in the treatment of infective lesions.

Acknowledgments

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The radiation of the chemical substances was made in the X-ray Department of the Toronto General Hospital by permission of Dr. G. E. Richards.

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COLLECTING AND HANDLING MOSQUITOES ON WESTERN EQUINE ENCEPHALITIS INVESTIGATIONS IN MANITOBA[1]

By J. McLintock[2]

Abstract

Mosquitoes used for virus analysis in Manitoba are obtained by means of hand catches or by the use of light traps, sometimes supplemented with dry ice. A trap is described that has been used successfully for this purpose during the past three years. This trap is simple to operate, the insects are taken in good condition for identification, and newly emerged specimens are killed, thereby eliminating the majority of those that could not possibly be infected with the virus of western equine encephalitis. The methods of shipping, sorting, identifying, and storing specimens are given. These methods are, in large part, standard entomological procedures adapted to present needs.

During the past four years the Manitoba Department of Health and Public Welfare, in conjunction with the Children's Hospital of Winnipeg, has carried on an investigation into the possible role of vectors in the spread of western equine encephalitis in the province. Thus far, attention has been concentrated on mosquitoes as the most likely suspects. In order to discover whether these insects are carrying the virus in nature it has been necessary to obtain large numbers of living specimens for virus analysis. For this purpose a trap was required that would attract a fairly large sample of the mosquito population, that would keep the mosquitoes alive until they reached the laboratory (for 24 to 48 hr.), and in good condition for identification. In addition, since the traps would be serviced by laymen, their operation had to be as simple and convenient as possible.

Two traps for a similar purpose have been designed by Reeves and Hammon (7) but these were intended to be operated under somewhat different conditions from those existing in Manitoba. One of their traps utilizes a light and carbon dioxide vapour as attractants, but the light was restricted to a 40° angle and in the morning the entrapped mosquitoes had to be removed by hand while the trap was still in the field. The other was a modified New Jersey trap in which the killing jar was replaced by a 14 in. square cage with the addition of a dry ice stimulus. Regarding this latter trap, the authors point out that, beside a fairly high mortality (36.2% compared with 20 7% in the "live trap"), "a large proportion of the 'living mosquitoes' in the standard trap were unsatisfactory for feeding experiments or other biological work, and their period of survival was often times brief". I believe that the trap described below has overcome most of the preceding difficulties, at least for the temperate climate of Canada. Dry ice can also be used with this trap and as is well known (3, p. 10; 7) greatly increases the proportion of females in the catch.

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Contribution from the Virus Laboratory of The Department of Health and Public Welfare for the Province of Manitoba and of the Children's Hospital of Winnipeg, Winnipeg, Man.

[2] *Entomologist.*

In the early stages of this investigation a modified version of Essig's (1) gnat trap was tried. In this trap an electric light attracted the insects and an electric fan drew them down into a cloth bag held distended by a wire frame. Although it attracted, trapped, and held the mosquitoes, and was simple to operate, this trap had the serious disadvantages that a large proportion of the specimens were bruised beyond recognition and the mortality was too high, sometimes reaching 60%. This was due to the specimens having to pass through the blades of a rotating fan on their way into a bag in which the resting surface was too small for the larger catches and which could not be protected from a certain amount of buffeting by the wind nor from heavy rains. After further experimentation to circumvent these difficulties, and following some suggestions made by Dr. W. McD. Hammon, the trap described below was designed and found to be quite satisfactory.

Since viruses will only propagate in living tissues, the problems of handling those tissues in such a way as to retain the virus with a sufficient titre for experimentation and of keeping the tissues susceptible to the virus, become of major importance in any investigation involving the aetiology of virus diseases. This fact has led the numerous authors of the growing literature on equine encephalitis to describe in some detail their methods of handling infected and possibly infected material. In those papers dealing with the role of mosquitoes in the transmission of the virus encephalitides it so happens that the investigations have either been carried out in climates considerably warmer than that of central Canada (the United States, Japan, Africa) or the reports have been published in the relatively inaccessible Russian literature. In central Canada it has been our experience that many of the methods used in warmer climates are wholly unsuited to conditions prevailing in the Prairie Provinces. As far as I am aware there is no previous report of living mosquitoes, taken in light traps, having been shipped to the laboratory for identification and preservation. Since this procedure has had to be followed in Manitoba during the past four years, it seems advisable to communicate the methods that have been successful in detecting infected mosquitoes in nature (6). No claim to originality is made for the entomological procedures involved, most of them having been borrowed from practices followed in other lines of entomological investigation and adapted to present needs.

The Trap and Its Operation

In so far as this trap utilizes an electric light to attract the insects and the suction of a fan to draw them in, it can be considered as a modified New Jersey trap. In the course of this investigation six of these traps have been located at different points in the province and, apart from minor mechanical breakdowns, have been in continuous operation during three seasons.

The trap assembly (Fig. 1) consists of three separate units, (1) a cylinder containing an electric motor and fan, (2) a funnel and canopy carrying a light, and (3) a cage that fits into the cylinder and holds the entrapped insects. The cylinder, of No. 24 gauge sheet iron, is about the size of a small garbage

can 23½ in. high and 12 in. in diameter. This cylinder is supported at a height of 10½ in. above the ground by three wooden legs bolted to its wall. The bottom 9¾ in. of the cylinder is occupied by a 1/15 or 1/4 h.p. electric motor carrying

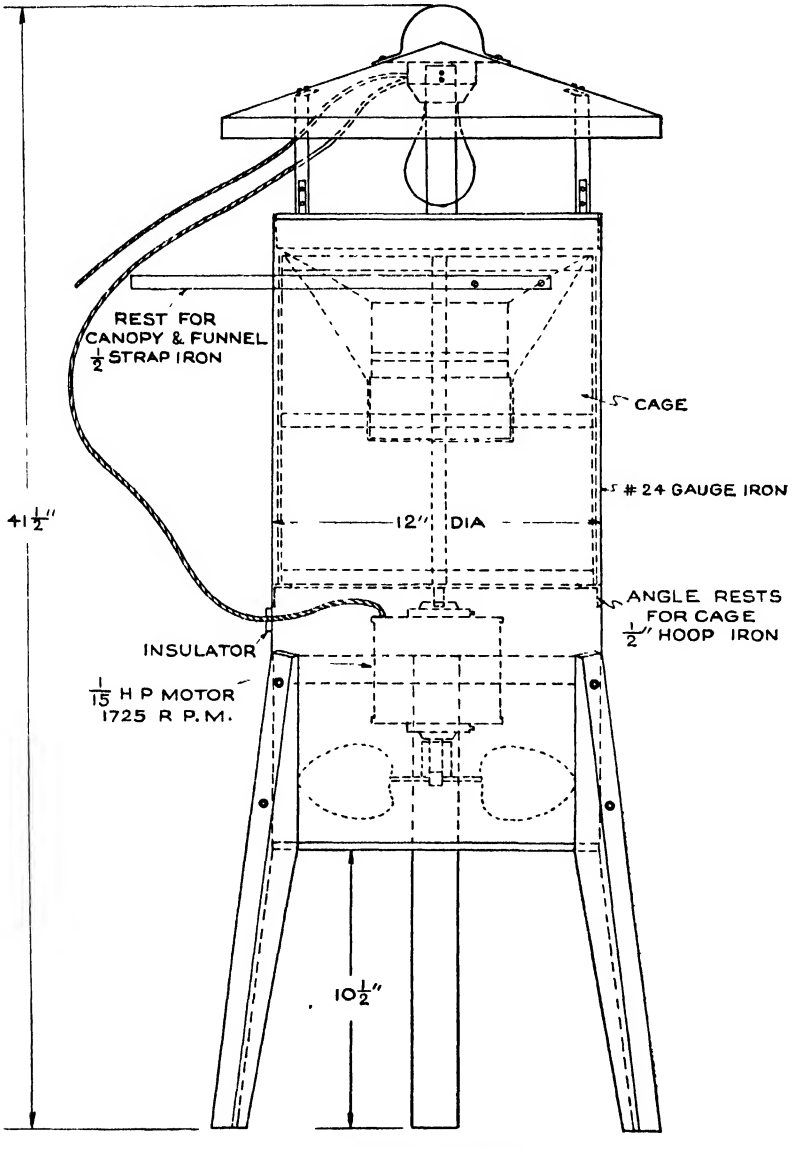

FIG. 1. *The trap assembly.*

a 10 in. exhaust fan, which it rotates at approximately 1725 r.p.m. The funnel (Fig. 2), of the same gauge iron, is made to fit into the top of the cylinder. This funnel tapers to a collar 5 in. in diameter and 2¾ in. long. A metal, cone-shaped canopy 16 in. in diameter covers the whole trap and is

supported by three legs of $1\frac{1}{4}$ in. hoop-iron spot-welded to the wide rim of the funnel and to the roof of the canopy. This places the bottom rim of the canopy $2\frac{3}{4}$ in. above the funnel and the top of the cylinder. A standard lamp socket to carry the light is attached to the inside of the apex of the canopy.

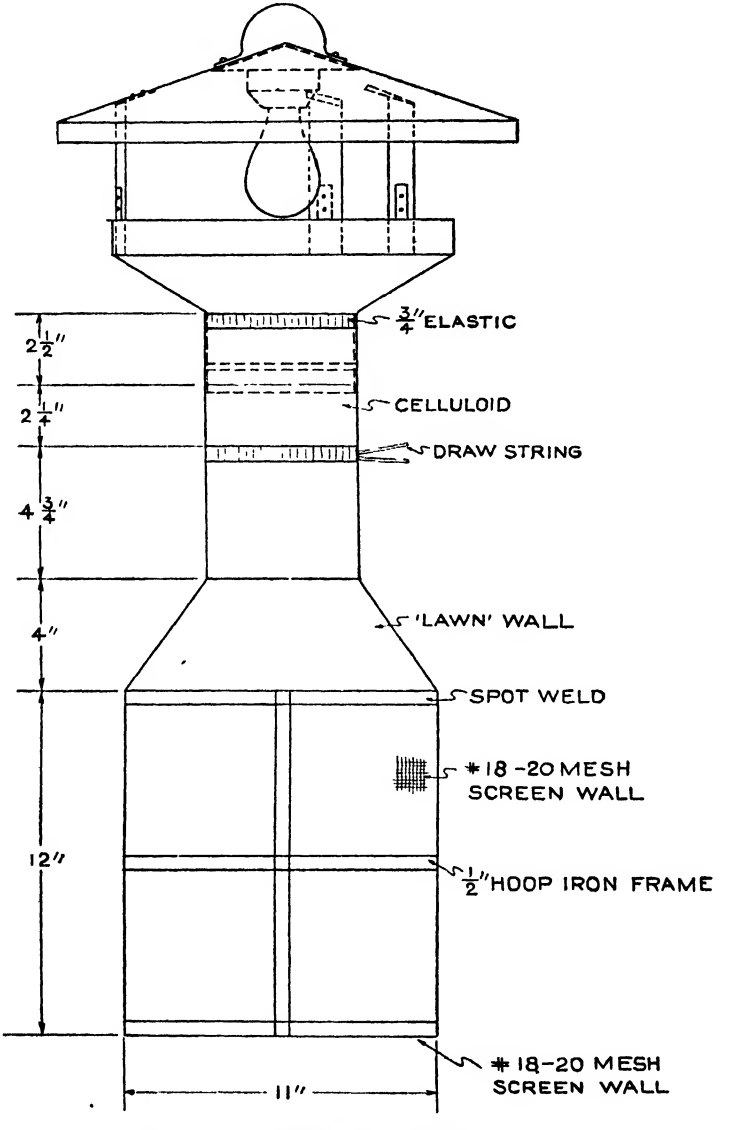

FIG. 2. *Sleeve of cage extended and attached to funnel and canopy.*

The canopy, lamp socket, and funnel form one unit, and the wires connecting lamp and motor in parallel are left long enough to allow this unit to be removed from the top of the cylinder by means of a handle attached to the top of the canopy. The top of the funnel when in position is held flush with the top

of the cylinder by means of three ½ in. hoop-iron cleats attached to the legs that support the canopy.

The body of the cage (Fig. 2) is a cylinder of 18-mesh galvanized or "galvanoid" wire screening, 11 in. in diameter and 12 in. high. The screening is supported on a ½ in. hoop-iron frame consisting of three horizontal circles and four vertical strips and also covers one end of the cylinder. The seams where the screening joins are soldered. The top of the cage is made of the cotton material known as 'lawn', which is sewn to the top rim of the screen cylinder from where it tapers for four inches to a diameter of 5¼ in. and from there on is continued as a sleeve 9½ in. long. At the end of this sleeve a ¾ in. elastic collar is sewn into the cloth and 2½ in. below this a celluloid collar 2¼ in. wide is also sewn into the cloth. A draw-string is inserted just below the celluloid. This construction allows the lawn sleeve to be 'telescoped' into the body of the cage. In the telescoped position the celluloid collar is near the bottom of the cage and is rigid enough to keep the aperture open. The draw-string permits the cage to be closed before removal from the trap and the elastic band attaches the lawn sleeve to the 5 in. collar of the trap funnel.

To set the trap, the funnel and canopy are removed and the cage placed inside the trap cylinder; three metal cleats support the bottom of the cage just above the fan motor, which places the top of the cage about 1½ in. below the top of the cylinder. The cage is then attached to the trap funnel by means of the elastic band at the end of the cage sleeve and the funnel placed in the top of the cylinder. In so doing the cage sleeve is telescoped into the cage. The current is then turned on. The insects are attracted by the 50 watt light under the canopy and are drawn down into the cage by the suction created by the fan. The telescoping arrangement of the sleeve provides an eddy-space at the top of the cage where the smaller insects such as mosquitoes can rest without being damaged by the larger insects that are drawn into the trap with them. It should be noted however that the suction of the fan is not strong enough to draw in insects any larger than cutworm moths or June beetles.

In the morning, a wad of rags is stuffed into the mouth of the funnel to prevent any of the entrapped insects from escaping when the fan motor is stopped. The funnel and canopy are then raised and rested on the edge of the cylinder while the draw-string is being pulled tight to close off the cage. The cage can then be lifted from the trap.

To obtain best results with the trap it is important that it be stopped and emptied as soon as possible after daybreak as the heat of the sun raises the temperature inside the trap with a consequent fatal lowering of the relative humidity. For this reason too the traps should be placed in locations that are shaded as much as possible from the rays of the rising sun. A 10 in. exhaust fan at 1725 r.p.m. moves about 150 to 200 cu. ft. of air per minute through a 5½ in. duct. This was found to have a considerable desiccating effect on the male and newly emerged mosquitoes. Mortalities as low as 17% (out of 1770 specimens) and 18% (out of 2460 specimens) were obtained for female mos-

quitoes under the best conditions of operation using only the light as attractant. During three successive nights when the weather was cloudy and cool and the light was supplemented with dry ice, 2862 specimens (all females) were obtained with a mortality of only 0.7%. However the mortality never went below 49% (out of 851 specimens) for the males. The mortality among newly emerged mosquitoes was determined by running the trap for several nights beside a slough during an emergence; here the mortality was 81% of both sexes combined (out of 327 specimens) and this was during a cool wet week. This selectivity is a decided advantage in collections made for the purpose of obtaining specimens for virus analysis. Only those specimens that have had a blood meal will be carrying virus and by the time a mosquito is ready to feed, its integument is already hardened and relatively impermeable. Since only living specimens are preserved or used for virus analysis, the trap eliminates a large number that would not be carrying virus.

In using dry ice, about 5 or 6 lb. are placed in a cloth bag and tied to the top of the canopy of the trap. The gas falls over the edge of the canopy and is drawn through the trap to be blown out at the bottom by the fan. An almost pure catch of female mosquitoes is obtained when dry ice is used.

Although this trap was designed primarily for trapping mosquitoes, it could obviously be used with equal success for trapping living specimens of other small insects that are attracted to light.

Hand Collecting

Hand collections are made by means of the aspirator described by Moore (5) or with the collecting bottle described by Mail (4). Suction for using the aspirator in the field is supplied by the intake manifold of a car and about 100 ft. of rubber tubing is sufficient to reach the inside walls of barns, sheds, stables, root houses, and other resting places. The amount of suction is controlled by means of a screw clip attached to the tubing about one foot from the aspirator. The collecting bottle is used only for picking the mosquitoes from horses and cattle in locations that cannot be reached by car. For collecting mosquitoes the aspirator has the advantage of holding a larger number of insects than the usual tube operated by mouth suction, also there is no possibility of the mosquitoes escaping once drawn in. When the storage tube of the aspirator, or the bottle, is full, it is emptied into the trap cage described above; each cage contains the collections from only one locality.

Shipping, Sorting, Identification, and Preservation of Specimens

The traps are serviced mainly by volunteer workers who send in the cages containing each night's catch, with a card showing the collection data, to the laboratory in Winnipeg. The cages are shipped via railway express in cardboard cartons 12 × 12 × 14 in. The bottom of each carton is lined with heavy waxed paper on top of which sits a 1 in. cellucotton pad kept moist with water. The cage rests on top of the pad. By this means the catches reach the laboratory never more than 48 hr. after being removed from the

traps. Counts made on hand catches shipped by this method have indicated that not more than 5% of the mortality is due to this procedure.

On arrival in the laboratory each cage is removed from its container, given a code number, and the collection data entered in a log book opposite the code number.

When a cage is held under a light the mosquitoes inside are easily seen through the wire screening and are removed from the cage by means of the aspirator mentioned above. In this case the aspirator is merely inserted through the sleeve of the cage. In the laboratory, suction for the aspirator is supplied either by a filter pump or by a standard laboratory vacuum pump. While the aspirator is held in one hand the rubber tubing is pinched in the other and only opened to pick up a mosquito. When the storage tube is full the aspirator is removed from the cage and disconnected from the rubber tubing. A finger is then placed over the end of the glass tubing to which the rubber tubing was attached and two or three drops of ether poured down the intake tube of the aspirator, care being taken that no liquid ether enters the storage tube. In a few minutes, when the insects have ceased moving, they are emptied into a 4 oz. pomade jar and covered with a piece of marquisette. A small wad of cotton and a vial of ether are kept handy on the bench where the identifications are being made and if the mosquitoes show signs of becoming active before they are identified, the cotton soaked with ether is held over the jar for a few minutes.

While still under the ether the mosquitoes are removed from the jar with forceps and identified under a microscope. As each specimen is identified it is placed in a 10 cc. ampoule, which is used for storage. As a rule only one species from one locality is placed in each ampoule and no less than 30 specimens to an ampoule. When the catches are small or contain a few specimens of the rarer species, either the same species from different localities are pooled or each of these species is isolated in jars covered with marquisette and containing a moistened pad of cellucotton; these are held until sufficient specimens are obtained to make up a quota for an ampoule. The stem of a 10 cc. ampoule is large enough to permit easy entry of a relaxed, anaesthetized mosquito, but too small to allow a flying mosquito to escape. When each ampoule has received its quota, it is sealed by quickly drawing out the stem in the fine flame of a blast burner, then given a code number and placed in a flat cardboard box lined with cotton. These boxes are stored on top of a block of dry ice kept in an ice chest.

For some time all living female mosquitoes taken were preserved intact for virus analysis. However Hammon, Reeves, and Izumi (2) have suggested the possibility that the comparatively fresh blood in the abdomens of newly engorged specimens might contain antibodies in sufficient titre to neutralize the virus in an inoculum prepared from wild-caught mosquitoes. Recently we have adopted their practice of crushing the abdomens of such specimens for smears for the precipitin test, the remainder of such engorged specimens being added to the non-engorged.

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RECOVERY OF A STRAIN OF WESTERN EQUINE ENCEPHALITIS VIRUS FROM *CULEX RESTUANS* (THEO.) (DIPTERA: CULICIDAE)[1]

By Marjorie Norris[2]

Abstract

Since an epidemic of western equine encephalitis in Manitoba in 1941, attempts have been made each year to isolate the virus from mosquitoes collected during the summer months. In 1942, 94 pools of mosquitoes were tested: 43 of the genus *Aedes*, 36 of *Culex*, and 15 of *Culiseta*. Suspensions made from each pool, which consisted of from 1 to 30 insects, were injected intracerebrally into guinea pigs. No isolation of virus was made. In 1943, 69 pools were tested: 49 of *Aedes*, 6 of *Culex*, 1 of *Culiseta*, 1 of *Anopheles*, and 12 of mixtures of different genera. Each suspension was derived from 10 to 75 mosquitoes and was injected intracerebrally into Rockefeller strain Swiss mice. There was no recovery of virus. In 1944, 90 pools were tested: 10 of *Culex*, 74 of *Aedes*, and 6 of *Culiseta*. Suspensions were usually made from 70 to 100 insects, and injected intracerebrally into Swiss mice of the Rockefeller strain. From one pool of *Culex restuans* (Theo.), a strain of western equine encephalitis virus was recovered.

Since Kelser (16) in 1933 showed that the mosquito *Aedes aegypti* could transmit the virus of western equine encephalitis under laboratory conditions, numerous other investigators have reported laboratory transmissions of this virus by other species of the genus *Aedes*. Their work has been summarized by Hammon and associates (12). The first report of the recovery of the virus from a naturally infected *Aedes* mosquito has been made recently by Hammon and Reeves (10).

Previous to 1941 *Culex* mosquitoes had not been implicated as vectors of equine encephalitis viruses. In that year Hammon and associates (14) reported the isolation of one strain of *W.E.E.** virus from *C. tarsalis* Coq. collected in the Yakima Valley, Wash., where annual epidemics of the disease occur. Shortly afterwards the recovery of four additional strains from the same species of mosquito was reported by the same group of workers (13). Later Hammon and Reeves proved that this species could act as a vector under experimental conditions (11). Transmission was also reported with *Culiseta inornata*, a mosquito that these workers had also found infected in nature.

The presence of *W.E.E.* virus in Western Canada was established. Fulton (3) had first isolated the virus from the brain of a horse during the epidemic of 1935 in Saskatchewan. In 1938, the same investigator (4) recovered this virus from a human brain during an epidemic amongst humans and horses in that province. In Alberta, Gwatkin and Moore (6) had shown the possible

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* *W.E.E. = western equine encephalitis.*

presence of *W.E.E.* virus in the brains of ground squirrels. Later Gwatkin (5) and Gwatkin and Moynihan (7) reported an unsuccessful search for carriers of equine encephalitis virus in a number of small mammals, birds, and a few insects.

Interest in the role of the mosquito as a possible vector of *W.E.E.* in Manitoba began in 1941 when the first epidemic of this disease in man in this province occurred, 509 cases being reported. Adamson and Dubo (1) reported their findings in 212 clinical cases. Serological tests were done on 125 of these, 55 being found to contain antibodies against *W.E.E.* virus. The etiological agent was further proved by the recovery of the virus from the spinal fluid of one patient (2). The same summer, during an epidemic in Alberta, Gwatkin and Moynihan (8) recovered a similar virus from the spinal fluid of a patient.

Following the epidemic in Manitoba, Mitchell and Pullin (17) made a survey of persons who had shown no clinical evidence of encephalitis. Their report showed that approximately 19% of blood specimens from 1013 persons contained neutralizing antibodies to *W.E.E.* virus.

In 1942 it was decided to attempt to isolate this virus from naturally infected mosquitoes. This report deals with the work from 1942 to 1944.

1942

Twenty-seven cases of encephalitis and 14 suspect cases were reported in the province. Complement fixation tests were done in this laboratory on blood specimens from all patients. Two were positive. Eighteen of the negative specimens were sent to Dr. J. Casals of the Rockefeller Institute, New York, who reported negative complement fixation tests for *W.E.E.* virus in all cases. Two negative specimens were sent to Dr. Herald R. Cox of Rocky Mountain Laboratory and six others to Dr. Wm. McD. Hammon of the University of California Medical Centre, for neutralization tests. With the exception of one specimen reported doubtful by Dr. Hammon, none was found to contain neutralizing antibodies for *W.E.E.* virus.

Mosquitoes were collected as described elsewhere*. They arrived at the laboratory alive and were identified and separated according to species before being made into suspensions for animal inoculation. Suspensions were made from 1 to 30 mosquitoes in 2.5 cc. of sterile normal saline. Each suspension was injected intracerebrally into two guinea pigs.

At first, in order to free the mosquitoes from bacteria, they were treated with ether for half an hour. It was soon found that suspensions made from mosquitoes artificially infected and then subjected to ether treatment, failed to infect mice when injected intracerebrally in dilutions of 10^{-1} to 10^{-4}, whereas suspensions of other experimentally infected mosquitoes, not treated with ether, were fatal to all mice injected in similar dilutions. To remove the bacteria from the latter suspensions, they were centrifuged in an angle centrifuge at approximately 4000 r.p.m. for 20 min. Cultures on blood plates

* *McLintock, J. Can. J. Research, E, 24 : 55-62. 1946.*

showed them to be almost free of bacteria. Likewise when a mouse brain infected with *W.E.E.* was divided in two, one half, treated with ether, failed to infect mice, whereas the other half, untreated, killed all mice injected.

Following this discovery, bacterial contamination of all mosquito suspensions was reduced simply by centrifuging at 4000 to 5000 r.p.m. for 20 min. Very little difficulty due to contamination was encountered, owing, beyond doubt, to the small number of mosquitoes used for each suspension.

A total of 94 pools of mosquitoes was made for animal inoculation. These were composed of 38 pools of *Aedes vexans* (Mg.), 2 of *A. dorsalis* (Mg.), 3 of *A. flavescens* (Mul.), 34 of *Culex tarsalis* (Coq.), 2 of *C. restuans* (Theo.), and 15 of *Culiseta inornata* (Will.). Of these, 11 pools of *A. vexans*, 6 of *C. tarsalis*, and 1 of *C. restuans* had been treated with ether.

No recovery of virus was made. Since comparatively few cases of encephalitis were reported and only two of these gave positive serological tests, it is possible that few mosquitoes were infected that year. Of the 94 pools tested, 51 were of the genera most frequently found to be natural vectors, namely 36 of *Culex* and 15 of *Culiseta*. However, as few insects were used in the preparation of each suspension, the possibility of recovering a virus was greatly diminished: Hammon and associates (13) working with *Culex tarsalis* recovered one strain of virus from 386 mosquitoes taken during an epidemic. Since this article was written, Hammon, Reeves, and Galindo (Am. J. Hygiene, 42 : 299-306. 1945) have found an infection rate of 1 in 78 *C. tarsalis* taken during an epidemic period in Kern County, Calif., in 1943.

1943

In 1943, 10 cases of encephalitis were reported. Blood specimens were obtained from only four patients. Complement fixation tests for western equine virus done in this laboratory were all negative. Neutralization tests on these specimens gave one positive result, one doubtful, and two negative.

The mosquitoes were shipped alive to the laboratory. After identification the different species were placed in separate ampoules.

After visiting Dr. Hammon's laboratory*, it was decided to adopt the method described by him and his associates (15) for preparing mosquitoes for animal inoculation. The mosquito suspension is freed from contaminating organisms by being centrifuged in the angle head of an International multispeed attachment at 16,000 to 18,000 r.p.m. for 10 min. Unfortunately this multispeed attachment was not available during the summer collecting period, consequently it was necessary to store all mosquitoes to be used for virus analysis in the dry-ice chest until the work could be begun.

The work of animal inoculation was carried out in January and February, 1944, five to seven months after the mosquitoes had been stored. To prepare the suspension for inoculation, the ampoules of mosquitoes were removed from the dry-ice chest just before use. Suspensions of six pools, the capacity of the centrifuge, were usually made at one time. The ampoules were opened

* *I should like to express my sincere appreciation to Dr. Hammon for the privilege of studying, under his guidance, the methods used in his laboratory.*

and the contents turned out on gauze and washed with 25 to 30 cc. of sterile distilled water. They were then thoroughly ground in a mortar with alundum, and small amounts of 33% rabbit-serum broth were added till a total volume of 3.0 cc. had been used. The suspensions were centrifuged in the angle centrifuge at approximately 18,000 r.p.m. for 15 min. To hold the suspensions at as low a temperature as possible during this process, the angle head was kept in the refrigerator until used and approximately 3 lb. of dry-ice were placed in the metal casing of the centrifuge.

A portion of the clear liquid that lay under the usually fine surface layer of fat was removed by means of a Pasteur pipette, the end of the pipette being carefully inserted through an opening in the surface layer. Cultures of all suspensions were made on blood agar plates. In the majority of cases, they were found to be sterile; contamination, where it did occur, was very slight.

The experimental animal was changed to Rockefeller strain Swiss mice since Hammon and associates (15) had reported this strain to be more susceptible to western equine virus than guinea pigs. Under ether anaesthesia, five such mice*, three to four weeks old, were inoculated intracerebrally with 0.03 cc. of each suspension. The time required for the preparation of the suspensions and their injection into mice was 45 to 60 min. The mice were watched for 14 days for the development of symptoms of encephalitis before being destroyed.

Sixty-nine pools of mosquitoes were tested. These consisted of 47 pools of *Aedes vexans*, 1 of *A. campestris* (D.K.), 1 of *A. flavescens*, 6 of *Culex tarsalis*, 1 of *Anopheles maculipennis* (Mg.) (= *occidentalis* D.), 1 of *Culiseta inornata*, and 12 of mixtures of different genera. Each pool contained 10 to 75 insects.

No virus was isolated. Failure to recover virus may indicate its absence from the mosquitoes tested: the almost complete absence of encephalitis in man and horses suggests that there was little virus 'on the wing'. This year, as in the previous year, the number of insects used for each suspension was often small. In addition, 49 of the 69 pools tested consisted of *Aedes* mosquitoes, which have rarely been found to be vectors in nature.

1944

Twelve cases of encephalitis were reported. Blood samples were taken from five patients. Three gave positive neutralization tests.

This year, as usual, with the exception of the one week mentioned below, the mosquitoes used for animal inoculation arrived at the laboratory in a living state. Suspensions for injection were usually derived from 70 to 100 mosquitoes. When the number of a single species in one collection from a district was considered sufficient for animal inoculation, suspensions were made and injected into mice at once, as described above. When there were not enough mosquitoes for immediate injection, the living insects were placed in ampoules, according to species, and frozen. When enough of any one

* *The mice from which our colony was bred, were very kindly sent to us by the late Dr. L. T. Webster of the Rockefeller Institute, New York.*

species had been accumulated in this way from a particular district, they were pooled, and the resulting suspension injected into five Rockefeller strain Swiss mice.

In the latter part of July, a small epidemic of encephalomyelitis in horses was reported in the neighbourhood of Portage la Prairie, a town 60 miles from Winnipeg, where no collections were being made. A trap was set up at once at a spot one-half mile distant from a farm where cases of the disease had occurred, and daily collections of living specimens were shipped to Winnipeg. To increase the number of mosquitoes that could be tested for virus, a temporary laboratory was set up in Portage la Prairie where further trap and hand collections were identified, stored in ampoules, and frozen. Daily shipments of these specimens, well packed in dry-ice, were sent to Winnipeg. They were picked up immediately on arrival and stored in ice-chest until their injection into animals, frequently the same day. Injection of all pools into experimental animals was completed by the middle of October.

Ninety pools were tested: 61 of *Aedes vexans*, 8 of *A. flavescens*, 1 of *A. dorsalis*, 1 of *A. campestris*, 1 of *A. spencerii* (Theo.), 1 of *A. excrucians* (Wlk.), 1 of *A. cinerius* (Mg.), 4 of *Culex tarsalis*, 6 of *C. restuans*, 6 of *Culiseta inornata*.

Suspensions of living insects were made from 20 pools: 14 of *Aedes vexans*, 3 of *A. flavescens*, 2 of *Culex restuans*, and 1 of *Culiseta inornata*. The remaining 70 pools consisted of frozen specimens.

Forty-one pools were derived from the epidemic area around Portage la Prairie: 23 of *Aedes vexans*, 7 of *A. flavescens*, 2 of *Culex tarsalis*, 5 of *C. restuans*, and 4 of *Culiseta inornata*. Of the above, 12 pools of *A. vexans*, 3 of *A. flavescens*, 2 of *C. restuans*, and 1 of *C. inornata* consisted of living specimens. The number of pools and the number of specimens tested are shown in Table I.

From one pool of *Culex restuans* consisting of 100 insects, alive until the preparation of the suspension, the virus of *W.E.E.* was isolated. These mosquitoes were from a trap collection received from Portage la Prairie. They were not engorged so that the possibility of the virus being derived from a recent feeding on an infected animal may be discarded. From the epidemic area, a total of 426 specimens of this species was tested.

Identification of the virus was begun when, after three serial mouse brain passages, intracerebral inoculation was made into two guinea pigs, an animal known to be susceptible to western equine virus. Both animals exhibited a weakness of the hind legs on the second day after inoculation. The temperature of No. 1 was not obtained, that of No. 2 was 104.4° F. On the third day the temperatures were 104.4° F. and 105.0° F. and definite paralysis of the hind quarters had developed in both animals. The brain of No. 2 was removed and stored in buffered glycerine.

Passage of a suspension of the brain of guinea pig No. 1 was made to two other guinea pigs, Nos. 3 and 4. On the third day after inoculation the temperature of No. 3 was 105.0° F. and No. 4, 104.1° F. There was no sign of muscular weakness. On the fourth day the temperature of No. 3 had dropped

TABLE I

MOSQUITOES TESTED FOR THE PRESENCE OF ENCEPHALITIS VIRUSES

Species	No. of pools from epidemic area		No. of pools from other areas		Total No. of pools	No. of specimens		Total No. of specimens
	Frozen	Living	Frozen	Living		Frozen	Living	
Aedes vexans	11	12	36	2	61	4605	1457	6062
Aedes flavescens	4	3	1		8	235	288	523
Aedes dorsalis			1		1*	47		47
Aedes campestris			1		1*	64		64
Aedes spencerii			1		1*	41		41
Aedes excrucians			1		1*	15		15
Aedes cinereus			1		1*	12		12
Culex tarsalis	2		2		4	241		241
Culex restuans	3	2	1		6	290	191	481
Culiseta inornata	3	1	2		6	438	95	533
Total	23	18	47	2	90	5988	2031	8019

* *These pools include specimens from the epidemic area.*

to 101.8° F. and paralysis of the hind legs was evident. The brain of this animal was removed and stored in buffered glycerine. On the same day the temperature of No. 4 had risen to 105.3° F., but there was still no sign of paralysis. This animal survived and its serum was later found to protect all mice against both dilutions of *W.E.E.* virus employed in the neutralization test.

The method used for the neutralization test was that described by Hammon and associates (9).

Titration of a 5% emulsion of our stock virus, the Rockefeller strain of *W.E.E.* virus, in 10% rabbit-serum broth, showed that a dilution of 10^{-6} was fatal to 95% of mice injected. This dilution and another 2×10^{-5} ($\frac{1}{5}$ of the higher) were the two dilutions used in the test.

Titration of the newly isolated virus was done after eight serial mouse brain passages, when the constant incubation period and the fact that all injected mice died, indicated that the virus was becoming adapted to brain tissue.

Neutralization tests were then run with four human sera known to contain neutralization antibodies to *W.E.E.* virus, normal rabbit serum being used as a negative control. One of these sera was from a resident of the district

where the epidemic amongst horses had occurred; he had developed encephalitis during this outbreak and had recovered. Two other specimens were from patients from widely separated districts in the province. They had recovered from attacks of encephalitis during the summer. The fourth was from a laboratory worker vaccinated against the virus. All four specimens protected all mice against both dilutions of virus used. Titration of the virus had indicated that a dilution of 2×10^{-5} of a 5% mouse brain suspension in 10% rabbit-serum broth caused the death of 95% of injected mice. This dilution and a second 4×10^{-4} were the two employed in the test.

As a final step in the identification, three adult guinea pigs, whose serum was shown to contain no neutralizing antibodies for the Rockefeller strain of *W.E.E.* virus, were immunized by intraperitoneal injections of suspensions of the newly isolated virus. Injections of 1.0 cc. of a suspension of infected mouse brain in broth in dilutions of 10^{-5} to 10^{-2} were given at weekly intervals. A week after the last injection of this series 0.5 cc. of 10^{-1} dilution was given. This was followed a week later by 1.0 cc. of the same dilution. The last injection was repeated twice. Ten days after the final injection, the sera of all pigs showed the presence of neutralizing bodies for Rockefeller strain of *W.E.E.* virus.

TABLE II

INTRAPERITONEAL INJECTIONS OF MOUSE BRAIN INFECTED WITH *W.E.E.* VIRUS, GIVEN AT WEEKLY INTERVALS

Dilution	Amount, cc.	Dilution	Amount, cc.	Dilution	Amount, cc.	Dilution	Amount, cc.
10^{-5}	1 0	10^{-3}	1.0	10^{-1}	0.5	10^{-1}	1.0
10^{-4}	1.0	10^{-2}	1.0	10^{-1}	1.0	10^{-1}	1.0

I believe that this completes the proof that the virus recovered from *C. restuans* caught in nature in Manitoba in the summer of 1944 was the virus of western equine encephalitis, the same virus as that recovered in this laboratory from a patient in the summer of 1941. Work on mosquitoes as carriers of this virus in Manitoba is continuing.

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MULTIPLE FEEDING HABITS OF SASKATCHEWAN MOSQUITOES[1]

By J. G. Rempel[2], W. A. Riddell[3], and Elspeth M. McNelly[4]

Abstract

Precipitin tests are used to determine the source of the blood meal of Saskatchewan mosquitoes. The tests disclose that the *Aedes* mosquitoes of the prairie feed extensively on man, horse, cow, and bird. The degree to which the species feed upon a particular host appears to be determined largely by the availability of that host. The number of individual specimens reacting to more than one antiserum is high, on the average one out of three belonging in this group. In spite of the multiple feeding habits of the *Aedes* species and their relatively great abundance, they do not appear to play a major role in the transmission of western equine encephalomyelitis.

During the summer of 1941 the province of Saskatchewan experienced a great outbreak of human encephalomyelitis. Although extensive outbreaks of equine encephalomyelitis preceded it in 1935, 1937, and 1938, there were comparatively few cases in man prior to 1941. In that year the number of diagnosed human cases reached 543, with 44 deaths. The greatest number of cases, i.e., 72.9%, occurred during the last three weeks of August, following an excessive outbreak of mosquitoes during the latter part of July and early August. Although cases of the disease were widely distributed over the province, the greatest incidence was found in an area encompassing Regina, Moose Jaw, and Weyburn.

Owing to the fact that on epidemiological grounds mosquitoes here and elsewhere were seriously suspected as being the vectors of the disease, a study of the species of the Regina district was undertaken in 1942 and continued through 1943 and 1944. During the first two seasons the general ecology of the species was stressed (12), while in the last summer emphasis was placed on feeding habits as revealed by field observations, and by means of the precipitin test. It was felt especially desirable to determine to what extent individual mosquitoes feed on more than one host, for, barring hereditary transmission, multiple feeding by the vector is a requisite to an epidemic of an insect-borne disease.

The precipitin reaction has been frequently used in studies connected with the feeding habits of insects after its original suggestion by Uhlenhuth, Weidanz, and Angeloff (13).

In view of the fact that a number of recent workers (1, 3, 4, 5, 11) had reported favourably on the use of this method it was decided to apply it in the investigation of the blood meal of mosquitoes.

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This investigation is part of a co-operative survey of encephalomyelitis in Saskatchewan by the Provincial Department of Public Health, the Federal Department of Agriculture, and the Departments of Veterinary Science and of Biology of the University of Saskatchewan.

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Laboratory Methods

The Preparation of Antiserum

In view of the fact that Saskatchewan is mainly an agricultural province, it was considered that mosquitoes would feed almost entirely on man and the domestic animals. Antisera were therefore prepared against human, equine, bovine, and avian serum. Human serum was obtained from the blood samples submitted for luetic serological examination, equine was purchased, while bovine and chicken blood were secured from the abattoir. Chicken serum was used as the representative of the avian group and in all cases filtered pooled sera were used.

The method of preparing the antiserum was essentially that given by Bull and King (3).

Fully grown, healthy, male rabbits were selected, and these were given a series of four intravenous injections of 5 cc. of serum on alternate days. A trial bleeding was made 10 days after the last injection, and the titre established. Titres in all cases except one were higher than 1:6400. The rabbits were starved for 18 hr., and then bled by cardiac puncture, about 30 cc. being obtained. The animals were bled again 48 hr. later. The rabbits were given a rest of one week, and the course of injection repeated.

In order to avoid anaphylactic reactions in the second series of injections, the rabbits were first given 1 cc. of serum subcutaneously. The following morning 1 cc. of serum diluted with 4 cc. of normal saline was given intravenously. In the afternoon 5 cc. of serum diluted with an equal amount of saline was given intravenously. This last injection was repeated three times at two day intervals. In spite of these precautions, it was found that rabbits that were injected with human serum suffered considerable shock during this second series of injections, although death did not occur. This response was not observed in the case of those injected with equine, bovine, and avian serum. Cardiac bleeding was done as before, but on the second bleeding the rabbits were killed and bled out aseptically.

The blood was allowed to clot and the serum was centrifuged. Fifty per cent of neutral glycerine was added as a preservative. This method was adopted after some experimentation with other methods of preserving the serum. 'Loading' small test-tubes was easier with a solution of greater density, and 'ring tests' were easier to read and not as easily dispersed as those of antisera preserved in other ways. All antisera were tested for absence of cross reactions before use.

The Collection and Preparation of Specimens

Gorged specimens of mosquitoes were identified in the field and the blood meal was pressed out on a good quality of analytical filter paper. Each droplet was numbered with a key number representing the species, and with a specimen number. A corresponding data sheet accompanied each set of specimens. A number of specimens in which the final feeding was known to the collector was submitted.

The filter paper was cut as close to the edge of the blood drop as possible and this small disk was placed in a ⅝ in. test-tube, which was then corked. Until the time of examination these were kept dry. When a series was to be examined, 0.5 cc. of saline was added to the disk of filter paper giving approximately a 1 : 50 dilution. The tubes were re-corked and placed in the refrigerator overnight. They were then ready for examination on the following morning. Experiments proved that this overnight extraction gives clearer reactions than extraction for a shorter time at room temperature, while a longer period of extraction gives cloudy and unsatisfactory results. Positive and negative controls were set up with each group of specimens.

Effect of Time

Table I indicates the readings obtained from specimens that were killed at various intervals after known human feedings. There was no observation or control of previous feedings. It appears that up to 18 hr. there is little difference in sensitivity or specificity of the reaction as far as can be judged from the small number of cases examined. For this reason also it is not possible to state whether there were more negative reactions or missed reactions as longer holding periods were used.

TABLE I

RESULTS OBTAINED FROM MOSQUITOES KILLED AT VARIOUS TIME INTERVALS AFTER KNOWN HUMAN FEEDINGS

Interval between feeding and killing, hr.	Simultaneous reactions				Multiple reactions in order of appearance				Other reactions			No reactions	Total specimens
	H	*HE*	*HB*	*HA*	*HE*	*EH*	*BH*	*EAH*	*E*	*B*	*A*		
1–4	—	—	2	1	1	2	1	—	—	—	—	—	7
5–9	21	5	2	3	1	2	—	1	3	2	1	2	43
10–14	14	—	—	—	—	—	—	—	1	—	—	2	17
15–18	8	1	—	—	3	—	—	—	—	—	—	1	13
Total	43	6	4	4	5	4	1	1	4	2	1	5	80

NOTE: *H = human; E = equine; B = bovine; A = avian.*

In the series of mosquitoes that were known to have fed on the collector, it is interesting that of the 80 specimens examined, 68 showed human reaction, five showed no reaction, and seven showed reaction other than human. Of those giving human reaction, 25 gave at least one other reaction, and of these only five showed the human first, while 14 occurred simultaneously, and in six cases another reaction occurred prior to the appearance of the human reaction.

A number of specimens were collected during the summer of 1943 and were not examined until June 1944 and during this time the blood meal had been stored as dried droplets on filter paper. There was apparently a higher proportion of negative tests but otherwise the results appeared to be quite satisfactory and the readings quite clear.

Method of Performing and Reading the Test

After some preliminary investigation the following procedures were adopted.

Equipment

(*a*) Small test-tubes, 4 mm. × 40 mm. (four tubes for each specimen). Cleaning these small tubes proved to be a time consuming task. It was necessary to load each one with cleaning fluid, and to add the distilled water for rinsing in the same way.

(*b*) Small test-tube racks to fit the above. These were made from masonite in our own laboratory. Each had two rows of 10 holes, which were staggered to facilitate reading. Thus two racks were required for each set of 10 specimens.

(*c*) Capillary pipettes with long tips.

Method

Tubes of Row No. 1 were loaded with one drop of glycerinated human antiserum, using a capillary pipette to deliver the serum to the bottom of the tube. The second row of tubes was similarly loaded with equine antiserum, the third with bovine, and the fourth with avian.

The first tubes of antiserum in each row were stratified with one drop of extract from the first specimen, the second tubes with the second specimen and so on. It was found that, allowing the necessary time for reading between incubations, 40 specimens were all that could be handled satisfactorily at one time.

Reading

The tests were incubated at 37° C. for 10 min., when they were removed and the first reading was made. Readings were made before a slit lamp.

The racks were returned to the incubator, and further readings were made at 20, 30, 45, and 60 min. intervals. No fresh reactions were observed in this series after the 30 min. reading and, at the end of 60 min., most of the reactions had faded out.

Species Studied

The species studied in this survey are those most abundant in the district (12). Some of these, namely, *Aedes vexans* (Meigen), *A. dorsalis* (Meigen), and *A. nigromaculis* (Ludlow) have been shown elsewhere to be capable of transmitting the virus of encephalomyelitis to laboratory animals (6). *Culex tarsalis* Coquillett and *Culiseta inornata* (Williston) are not represented in the study because of the small numbers available during the 1944 season. This is regrettable because both species have repeatedly been shown by Hammon to harbour the virus in nature (7, 9), and also readily to transmit it to laboratory animals (7, 10). Moreover, the work of Hammon *et al.* lends strong support to the view that *Culex tarsalis* is the main vector of the disease (8).

A survey made in the Regina district in late August, 1941, showed that the species represented in the great mosquito outbreak that preceded the ence-

phalomyelitis epidemic were *Aedes nigromaculis*, *A. spencerii* (Theobald), *A. dorsalis*, *A. vexans*, and *A. campestris* (Dyar and Knab). Larvae of *Culex tarsalis* and *Culiseta inornata* were exceptionally abundant in late August of that year, suggesting that both species were well represented in the earlier outbreak.

Results and Interpretation

The results of the precipitin tests indicate that the *Aedes* species of the prairie feed extensively on man, horse, cow, and bird (Tables II and III). In spite of the fact that the numbers dealt with in this paper are small, it was decided to express them in the form of percentages. Although these results in general are in agreement with field observations on the feeding habits of the species, the number reacting to avian antiserum was felt to be surprisingly high (Table III). However, owing to the absence of species specificity in the case of avian antiserum, these specimens may have fed not only on chickens, but also on other domestic fowl and on wild birds. The number of specimens not reacting to any of the four antisera is low, suggesting that only on rare occasions is the blood meal obtained from a host other than one of the four mentioned above. *Aedes flavescens* (Mueller) is an exception. When collected in a habitat with few or no domestic animals in the neighbourhood (Table II (*a* and *c*)), almost one-fourth are negative, suggesting that some other animal, such as a gopher, may be the host. Of the species studied only *A. flavescens* will not readily migrate for a long distance from its breeding habitat in search of food. None of the species studied show a decided host preference. The degree to which the species feed upon a particular host appears to be determined largely by the availability of that host. Thus at the outskirts of the city the percentage of those that feed on man is higher than in the country. Also, mosquitoes collected near an Indian encampment at the outskirts of the city with several hundred horses grazing in the pasture, react in great numbers to horse and human antisera.

The number of individual specimens reacting to more than one antiserum is high, on the average one out of three belonging in this group. Frequently no mention of double positives is made in the literature. When they have been observed, it has been the custom, for statistical purposes, to call them negative, or to assign half value to each, or to call the first and stronger reaction positive (2), ignoring the other. However Badzhiev (1), in his work on *Anopheles* mosquitoes, records frequent multiple positives (almost 30% not counting those giving cross species reactions) and assigns equal value to all.

In the present work double positives were frequently observed and in most cases neither one nor the other reaction was more prominent. The difference, when observed, was merely one of time. However, we feel that the difference in the time of the appearance of the precipitate is dependent upon the concentration of the antigen at the interface between antiserum and extract. A high concentration at the interface will result in an immediate reaction, while in the case of a low concentration the reaction has to await the diffusion of antigen into the region of the interface. But this requires time.

TABLE II

FEEDING HABITS OF SASKATCHEWAN MOSQUITOES

Source of mosquitoes	Species of mosquitoes	Percentage of blood smears reacting to specific antisera														% Neg.	No. tested
		H	*HE*	*HB*	*HA*	*E*	*EB*	*EA*	*B*	*BA*	*A*	*HEB*	*HEA*	*HBA*	*EBA*		
(*a*) Collected over vegetation in pasture. No domestic animals present at time	*Aedes spencerii*	8 0	8.9	2 8	2 8	21.2	14 1	7 0	16 0	6 1	8 5	0 9	0.5		1.4	1.8	213
	A. flavescens	15 6	20 0	2.2	6 7	6 7	2 2	11 1	4 4	2 2	15 6	2.2				11.1	45
	A. vexans	*x*				*x*											2
	A. nigromaculis	21 3		7 1	7 1	7 1	14.4		14.4		14 4		7.1			7 1	14
	A. dorsalis																0
(*b*) Collected over vegetation in pasture near farm yard. Domestic animals near	*A. spencerii*	11 8	8 2	2 7	8 2	25 4	4.5	8 2	6 4	6 4	12.7			1.0		4.5	110
	A. flavescens	8 8	7 5	2 5	2 5	25 0	15.0	15 0	10 0	3 7	8.8				1.2		80
	A. vexans	12 4	4 2	4 2	4 2	14 5	8 3	4 2	16.7	8.4	14 5			2 1	4.2	2.1	48
	A. nigromaculis	24 0	12 0	2 4	5 2	21 0	6.0	6 0	9 8	1 6	5 2		0 8	0.8		5.2	133
	A. dorsalis		9 1			36 3	9 1		36 4		9.1						11
(*c*) Collected over vegetation near outskirts of city. No domestic animals nearby	*A. spencerii*	25 5	4 2	4 2	12 7	18 3	2.8	5.6	8.5	2 8	7 0	1 4		1.4	1.4	4.2	71
	A. flavescens	17 6	12 3	1 7	3 5	14 0	5.3	3 5	5.3	1 7	10.5			1.7		22.9	57
	A. vexans	18 8				18 7	6.3	6 3	15 6	3 1	21 8			3.1		6.3	32
	A. nigromaculis	11 1			11 1	11.1	22.2		11.1		22 3					11.1	9
	A. dorsalis	21 1	12 1	2 3	5 3	13 6	2.3	2 3	10 6	2 3	25.0			0.8		2.3	132
(*d*) Collected over vegetation near outskirts of city beside Indian encampment	*A. spencerii*	17 3	15 9	1 4	4 2	33 2	2 1	4 9	6.3	1 4	6 3		0.7			6.3	144
	A. flavescens	12 1	20 6	8 6		22 4	6 9		6.9		12 1	1.8				8.6	58
	A. vexans	28 8	5 3	2 7		28 8	7 9		7 9	2.7	7.9					8.0	38
	A. nigromaculis	30 0	10 0			20 0	10 0	10 0	20 0								10
	A. dorsalis	21 8	39 3	4 3	4.3	8 6		4 3	13.1		4.3						23
Total																	1230

NOTE: *H = human; E = equine; B = bovine; A = avian; x represents one specimen.*

TABLE III

FEEDING HABITS OF SASKATCHEWAN MOSQUITOES

Source of mosquitoes	Species of mosquitoes	Percentage of blood smears reacting to specific antisera				% Neg.,	No. tested
		Percentage of total reacting to human antiserum	Percentage of total reacting to equine antiserum	Percentage of total reacting to bovine antiserum	Percentage of total reacting to avian antiserum		
(a) Collected over vegetation in pasture. No domestic animals present at time	*A. spencerii*	23.9	54.0	41.3	26.3	1.8	213
	A. flavescens	46 7	42.2	13.3	35.6	11.1	45
	A. vexans	x	x				2
	A. nigromaculis	42 9	28 6	35.7	28.6	7.2	14
	A. dorsalis						0
(b) Collected over vegetation in pasture near farm yard. Domestic animals near	*A. spencerii*	31.9	46.3	21.0	36.5	4.5	110
	A. flavescens	21.3	63.7	32.4	31.2		80
	A. vexans	27.1	35 4	43 9	37 6	2.1	48
	A. nigromaculis	45.1	45 9	20 3	19 5	5.3	133
	A. dorsalis	9 1	54.5	45.5	9 1		11
(c) Collected over vegetation near outskirts of city. No domestic animals nearby	*A. spencerii*	58 5	33 7	22.5	30.9	4 2	71
	A. flavescens	36 8	35.1	15.7	20 9	22 9	57
	A. vexans	21 9	31 3	28.1	34 3	6 3	32
	A. nigromaculis	22 2	33 3	33 3	33.4	11.1	9
	A. dorsalis	41 6	30 3	18 3	35.7	2.3	132
(d) Collected over vegetation near outskirts of city beside Indian encampment	*A. spencersi*	39 5	56 8	11 2	17.5	6 3	144
	A. flavescens	43.1	51 7	24.2	12.1	8 6	58
	A. vexans	36 8	42 0	21 2	10 6	8 0	38
	A. nigromaculis	40 0	50 0	30 0	10 0		10
	A. dorsalis	69 7	52 2	17 4	12 9		23
Total							1230

To accept, then, the first reaction and ignore the second, appears completely to invalidate the use of the precipitin test. It should also be pointed out that on a number of occasions two reactions occurred *simultaneously*. One might well ask, then, which was to be considered positive. It should be stressed also that none of these double positive tests belonged to recognized cross-species reactions.

We interpret our results in the following manner. In the case of two reactions occurring simultaneously, the concentration of antigen at the interface was the same. The condition could obtain if blood in equal amounts were taken from two hosts with little time between the feedings, or if the first feeding was complete, followed after a considerable interval by a partial second feeding. Similarly, in the case of two reactions not occurring simultaneously, the first reaction may indicate the last feeding, or merely a greater blood meal. The tests, therefore, disclose the host or hosts, but do not reveal the order of feeding, nor the relative amounts of blood taken at each feeding.

If our readings reflect a true picture, then the *Aedes* species of the prairie cannot be looked upon as important vectors of encephalomyelitis. During the past few summers *Aedes* species have on occasion been very abundant in certain localities and on the basis of the precipitin test they fed extensively on more than one host. Yet the expected outbreak of the disease did not occur unless *Culiseta inornata* and *Culex tarsalis* were present. This indirect evidence incriminating the last two species as the main vectors of the disease is in complete agreement with the findings in California and elsewhere.

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THE REDUCTION OF HYDROGEN PEROXIDE BY FIBRIN[1]

By L. B. Jaques[2] and H. J. Bell[3]

Abstract

It has previously been reported by Jaques that fibrinogen and fibrin possess marked reducing powers. In a further investigation of this property, the reduction of hydrogen peroxide by these proteins has been studied. Commercial fibrin sometimes contains large amounts of catalase. Methionine in phosphate buffer reduces hydrogen peroxide at pH 6 6 and temperature of 25° C. This reduction is increased by copper and molybdate. The reduction of hydrogen peroxide by fibrinogen and fibrin free of catalase is due to the methionine residue.

Jaques (3) has previously reported that fibrinogen and fibrin show marked reducing properties at pH 6.6 and 25° C. While the reduction of iodine was traced to the aromatic residues (tyrosine and tryptophane), the reduction of hydrogen peroxide was not due to these amino acid groups. Cysteine was not a factor since these proteins fail to give a direct nitroprusside reaction, even after denaturation. Toennies and Callan (8) have shown that methionine is a stronger reducing agent for hydrogen peroxide than cysteine and we have investigated the contribution of methionine to the reaction previously reported. It appears likely that methionine is responsible for the effect observed. A further factor has been demonstrated, namely, that some commercial preparations of fibrin contain large amounts of catalase.

Materials and Methods

These were the same as in the previous study, the fibrinogen being prepared from horse plasma by a method similar to that of Florkin. In the preparation chiefly used, the horse plasma was subjected to a preliminary treatment with tricalcium phosphate. Commercial fibrin was obtained from British Drug Houses, Ltd. and Difco Laboratories, Inc. The protein and buffer, water, hydrogen peroxide, etc. were placed in glass-stoppered flasks in a constant temperature bath at 25° C. The reaction was stopped by adding potassium iodide and 4*N* sulphuric acid and the residual hydrogen peroxide determined by titration of the equivalent iodine with sodium thiosulphate. Measurements were made with the Beckman glass electrode.

Results and Discussion

The Catalase Activity of Fibrin

When a commercial preparation of Difco fibrin was tested for reducing power, it was found that it rapidly destroyed large quantities of hydrogen peroxide (Table I). Thus 100 mgm. of fibrin decomposed 10.5 ml. of *N*/10

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hydrogen peroxide in one hour and 54 ml. in 16 hr. This is many times the reducing power of fibrin previously studied and since it is equivalent to a reduction of 184 mgm. of hydrogen peroxide per 100 mgm. of protein, it must be due to a catalytic decomposition of the hydrogen peroxide and not to a simple reaction with a protein group. This was confirmed by collection of the oxygen in a spirometer.

TABLE I

THE DESTRUCTION OF HYDROGEN PEROXIDE BY DIFCO FIBRIN

Time	$N/10$ H_2O_2 destroyed, ml.*
11½ min.	2.18
37 "	4.88
60 "	10.49
91 "	11.57
120 "	15.62
16 hr.	53.70
30 "	54.66

NOTE: $H_2O_2 = 0.888\ M$.
* *By 100 mgm. fibrin.*

Experiments were conducted to determine the nature of the catalytic agent. Heating the fibrin suspended in buffer for five minutes at 100° C. destroyed its reducing power, with the exception of a small amount of residual reduction. The effect of incubation at 40° C. at various pH levels for one hour was studied, the reducing activity being tested at pH 6.6. Complete loss of activity occurred at pH 2.2 and 4.0 but no loss at 6.0 and some loss at pH 8.0. The addition of $M/400$ cyanide resulted in about 50% inhibition. It is probable, therefore, that the catalytic reduction of hydrogen peroxide is due to the presence of catalase in the fibrin preparation. It was found that the catalase is not always distributed uniformly through the sample, causing practical difficulties in studying this activity. Fibrin from British Drug Houses, Ltd. showed some catalase activity, although much less than the Difco fibrin. Thus, 100 mgm. decomposed only 0.8 ml. of $N/10$ hydrogen peroxide after 30 hr.

The presence of catalase activity in fibrin was first reported by Thenard in 1818 (cf. 2). It is of interest that an enzyme as unstable as catalase, when adsorbed on fibrin, survives the drastic procedures involved in the commercial preparation of the fibrin. The catalase probably comes from the red cells since the Difco fibrin, containing large amounts of the enzyme, is prepared by defibrinating whole blood.

The Non-catalase Reducing Power of Fibrin

After destruction of the catalase activity of fibrin, there still remains the reducing activity previously reported. It was found that this activity is

not destroyed by heat or by hydrolysis with hydrochloric acid, sulphuric acid, baryta, or trypsin. Qualitative tests for ferrous and ferric iron on the ash from fibrin were completely negative. Samples of fibrin and of fibrin oxidized with hydrogen peroxide under the conditions used were subjected to various colour tests. No difference between the oxidized and unoxidized protein was observed for the following reactions—biuret, Molisch, xanthoproteic, Millon, Adamkiewcz, Ehrlich, Sullivan and Hess, Sakaguchi, ninhydrin, and the lead acetate test for sulphur. However, the McCarthy and Sullivan test for methionine, which was strongly positive before oxidation, was completely negative afterwards. Hence the reduction must be, at least in part, due to the oxidation of methionine.

Toennies and Callan have studied the oxidation of methionine by hydrogen peroxide in acid media. They found that the reaction is accelerated by increasing the acidity to above pH 1.0 and that the oxidation of methionine was confined to the sulphoxide stage, but if molybdate or perchloric acid were added, the oxidation proceeded to the sulphone. In a preliminary experiment we found that methionine reduced hydrogen peroxide under the conditions used. This was further studied as shown in Table II.

TABLE II

FACTORS INFLUENCING OXIDATION OF METHIONINE BY H_2O_2

Buffer, etc.	pH	H_2O_2 reduced, ml. of $N/200$
HCl	0 90	1.037
HCl	2 35	0.859
Acetate	4.51	0.793
Acetate	4 73	0.568
Acetate	5.03	0.453
Acetate	5.62	0.130
Acetate + phosphate	5.47	0.617
Acetate + molybdate	4.93	1.052

NOTE: *Methionine* 2.68×10^{-3} *M.*
H_2O_2 *0.0365 M.*
Molybdate 4×10^{-4} *M ammonium molybdate.*
Phosphate *M/24 potassium phosphate, pH 6.6.*
Temperature *25° C.*
Reaction time *41 hr.*

As shown by Toennies and Callan, methionine reduces hydrogen peroxide in strongly acid solutions. However, reduction of hydrogen peroxide still occurs above pH 4, although it falls off rapidly between pH 4.5 and 5.6. Both phosphate and molybdate ions markedly increase the reduction. Hence it appears evident that while the reduction by methionine alone is negligible above pH 6.0, yet the presence of phosphate or molybdate will result in the measurable reduction found in the preliminary experiment. The reaction in phosphate at 25° C. and pH 6.6 reaches an equilibrium in 15 to 24 hr. with

50% reduction being reached after six hours. At equilibrium a linear relation was obtained between methionine concentration and hydrogen peroxide reduced.

Toennies and Callan reported that copper had no effect on the reduction of hydrogen peroxide by methionine. Since copper catalyses the reduction of hydrogen peroxide by cysteine (5, 7), this might be used to distinguish the reduction by cysteine and methionine. Table III shows the number of ml. of $N/200$ hydrogen peroxide reduced by 1 mgm. methionine in the presence of various reagents. Phosphate, copper, and molybdate markedly increase the

TABLE III

REDUCTION OF HYDROGEN PEROXIDE BY METHIONINE, FIBRIN, AND EGG ALBUMIN

Solvent	$N/200$ H_2O_2 reduced, ml.		
	Methionine, 1 mgm.	Fibrin, 8 33 mgm.	Egg albumin, 1 66 mgm.
Water	0 35	0.11	0 14
Phosphate buffer	0 59	0 23	0.25
Copper + water	1 72	3 17	2.40
Copper + phosphate	2 42	3 89	1.93
Molybdate + water	3 92	2 72	0.41
Molybdate + copper + water	4 73	4 16	3 15

H_2O_2 *0.043 M.*
Copper *1.3×10^{-3} M copper sulphate.*
Molybdate *$4\ 2 \times 10^{-4}$ M ammonium molybdate.*
Phosphate *M/24 potassium phosphate, pH 6.6.*
Reaction time *= 41 hr.*

reduction by methionine, the copper and molybdate being particularly effective. The effects of phosphate and copper and of molybdate and copper are additive. The presence of copper caused some difficulty in the titrations, however. As in the other experiments, the values reported show the increased reduction compared to suitable controls with methionine or protein omitted. Reduction in the control flasks was very small under the conditions used. The difference in the effect of copper from that reported by Toennies and Callan is presumably due to the quite different pH level used. Also shown are the results obtained with fibrin and egg albumin. The reduction by both proteins is increased by phosphate, copper, and molybdate. Egg albumin shows a marked increase with copper and a relatively smaller increase with molybdate, while the increased reduction with copper is decreased by phosphate. The results with fibrin resemble those with methionine, although the increase in the presence of copper is greater than would be expected on this basis. Toennies and Callan have previously suggested that the increased reduction of hydrogen peroxide by proteins in the presence of copper and molybdate is due in part to components other than methionine and cysteine.

In order to assess the contribution of methionine to the reduction of hydrogen peroxide by fibrin and fibrinogen, the methionine content of fibrinogen and fibrin was determined by the method of Kolb and Toennies (4). The values found were 2.95% in fibrin and 2.77% in horse fibrinogen. Brand, Kassell, and Saidel (1) report values for methionine, obtained by other methods, of 2.62 to 2.72% in fibrin and 2.52% in fibrinogen. The total reduction by the protein, the reduction after heating for five minutes at 100° C., and the reduction by the amount of methionine present was then determined for 40 hr. in phosphate buffer at pH 6.6. The reductions in ml. of $N/200$ hydrogen peroxide per 10 mgm. of protein were for fibrin, total reduction, 1.565; residual reduction after heating, 0.236; reduction by equivalent methionine, 0.252. No loss of reduction occurred after heating the fibrinogen. Reduction by fibrinogen was 0.273 and by the methionine, 0.251. The reduction by fibrin was equivalent to the reduction by the catalase plus methionine present, while the reduction by fibrinogen and the methionine it contained were the same, so it appears likely that these two factors are alone responsible for the reduction by these proteins. No consistent difference in reducing power between fibrinogen and fibrin has been found. It appears probable that a trace of catalase in the sample of fibrin previously used was responsible for the increased reduction by fibrin reported.

It is evident that the reduction of hydrogen peroxide by methionine under relatively mild conditions is a property also of methionine present in protein and may be of considerable significance in considering the properties of proteins. However, it does not appear to be of value for the determination of methionine in protein unless the protein is first hydrolysed, as in the method of Kolb and Toennies.

After we had completed this study, Perlman and Lipmann (6) published the results of a comprehensive study of the catalase in serum and plasma. They reported that slight haemolysis increased the serum catalase in proportion to the amount of haemoglobin released. However, normal sera contained approximately uniform amounts of catalase.

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THE SEROLOGICAL RELATIONSHIPS OF THE RICKETTSIAE OF EPIDEMIC AND MURINE TYPHUS[1]

By James Craigie[2], Dennis W. Watson[3], Eina M. Clark[4], and M. Elizabeth Malcomson[5]

Abstract

The complement-fixing antibodies present in epidemic and murine typhus immune sera can be differentiated by quantitative absorption tests. The neutralizing antibodies that participate in the Giroud reaction can be differentiated by quantitative inhibition tests.

Epidemic and murine typhus immune sera contain two kinds of complement-fixing antibodies and also two kinds of neutralizing antibodies. The antigens that react with these antibodies differ in specificity and thermal resistance. Cross reactions between epidemic and murine rickettsiae are due to the presence of similar heat stable antigens in the two types. Type specific sera may be obtained by absorbing immune serum with either (*a*) rickettsiae of heterologous type or (*b*) heated rickettsiae of homologous type. The specific antibodies react only with the heat labile antigens of the homologous type of rickettsiae.

Mice may be actively immunized against the toxic factors of murine and epidemic rickettsiae. The immunity produced by small doses of vaccine is type specific and dependent on the presence of heat labile antigen in the vaccine.

In the absence of data obtained from reciprocal cross absorption tests, any one of the following three hypotheses might be used to explain the serological and immunological relationships of murine and epidemic typhus rickettsiae.

1. The two types of rickettsiae share the same antigens in different proportions, i.e., each type contains a small amount of the antigen that characterizes the heterologous type.
2. The epidemic type is the murine type that has acquired additional antigens.
3. Epidemic and murine types of rickettsiae possess different type specific antigens and their observed cross immunological reactions are referable to an additional antigen common to both types.

If major importance is attached to the cross immunity that occurs between epidemic and murine typhus, and if Zinsser's (16) suggestion of transformation of the murine type to the classic epidemic type by repeated louse–man passage is accepted, it might seem to be unnecessary to postulate type specific antigens. Either the first or the second hypothesis might explain the cross

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immunity that develops after infection with one of the two types. The partial cross immunity of guinea-pigs vaccinated with killed rickettsiae could be explained by the presence of a relatively small quantity of heterologous antigen, but is somewhat more difficult to explain on the basis of the second hypothesis unless it is postulated that the additional epidemic antigen prevents adequate response to the murine component. Intermediate strains of typhus could be regarded either as having a more equal ratio of epidemic and murine antigen (first hypothesis), or as having less epidemic antigen that is found in typical epidemic strains (second hypothesis).

Zinsser and Castaneda (17) observed that although cross agglutination occurs between epidemic and murine rickettsiae, immune sera react to a higher titre with the homologous type. Van Rooyen and Bearcroft (15) have recently reported similar results obtained with convalescent sera from cases of epidemic and murine typhus. In the case of the complement fixation test, marked cross reactions may occur*; but Plotz (13) has reported on the use of rickettsial antigens that yield more specific results. Plotz carried out absorption tests on specimens of serum from cases of Brill's disease. Murine rickettsiae absorbed only the murine antibody, but epidemic rickettsiae removed antibodies for both murine and epidemic rickettsiae respectively. Plotz suggested that the epidemic antigen may be more complex than the endemic antigen. This view would be in keeping with the second hypothesis already mentioned.

Neither the first nor the second hypothesis takes into account the heat labile and heat stable antigens of rickettsiae. The most recent discussion of these antigens is that presented by Felix (7). Felix draws an analogy with the Vi and O antigens of *Bacterium typhosum* but does not enlarge on the differences between the rickettsiae of murine and epidemic typhus. Actually, the heat lability of certain rickettsial antigens provides one of the most important clues to differences in antigenic structure. The specific agglutinogens of epidemic and murine strains are labile at 56° C. It was this that led the senior author to use these agglutinogens as a guide in the development of ether processing methods that aimed at the retention of the maximum amount of labile antigen in the rickettsiae. More recently, a direct mouse test (4, 6) was investigated and adopted as an antigenic test for typhus vaccine prepared by the Cox method of culture and processed at pH 7.0 by the method described in Memo. No. 5 (5). It has been found that the protection conferred by this type of vaccine is highly specific and that the immunizing antigen is, like the specific agglutinogen, labile at 56° C. Such findings suggested that the third hypothesis, presupposing two type specific antigens, was more probably correct, but other observations indicated some further degree of antigenic complexity. For example, complement fixation tests failed to show a commensurate destruction of antigen in vaccines heated at 56° or 60° C. When the serological responses of guinea-pigs to unheated and heated vaccine were compared, there was no evidence that heating impaired the capacity of the vaccine

* *Unpublished observations.*

to stimulate complement-fixing antibodies or neutralizing antibodies (Giroud test).

It therefore became necessary to resort to complete reciprocal cross absorption tests*, utilizing the fact that some of the rickettsial antigens are heat labile. The results of a number of these tests are presented in the sections that follow**.

I. The Complement-fixing Antigens of Epidemic and Murine Strains

Materials

Strains of Rickettsiae

The following strains were used: (*a*) Breinl epidemic; (*b*) Madrid epidemic; and (*c*) Castaneda murine.

Guinea-pig Sera

These were obtained from guinea-pigs infected with yolk sac cultures. The animals were bled 14 to 21 days after the onset of fever. Sera from several guinea-pigs inoculated with the same strain were pooled and inactivated at 56° C. None of these sera contained demonstrable mounts of yolk antibody.

Antigens

(1) Soluble antigens. Ether processed vaccine stocks*** were centrifuged in an angle head at 4000 r.p.m. for 45 min. The supernatants, which contained the soluble antigens, were used in dilutions determined by preliminary titrations. In their undiluted state they contained approximately 0.02% formalin and 1 in 10,000 merthiolate.

(2) Washed rickettsiae. The rickettsiae that had been separated from the soluble antigen by centrifugation were suspended in saline containing 0.5% formalin and subjected to further purification with ether. The suspensions were used for the titration of complement-fixing antibody in dilutions determined by preliminary titrations. The dense suspensions used for absorbing the sera were 30 to 50 times more concentrated than this.

Methods

Complement Fixation Tests

The method of Plotz and Wertman (14) was used with the reagents described.

Absorption Tests

The required amount of the absorbing suspension of rickettsiae was centrifuged in an angle centrifuge, the supernatant was decanted, and the remaining drops of fluid were removed with strips of sterile filter paper. The serum that was to be absorbed was intimately mixed with the deposit of rickettsiae in a dilution of 1 in 6.25. After being kept at room temperature for two hours,

* *Since the experimental work described in this memorandum was undertaken, two reports (1, 11) containing data on the results of cross absorption tests have been received (see ensuing discussion).*

** *The complement fixation tests were undertaken by M.E.M., the modified Giroud tests by D.W.W., and the mouse tests by J.C. and E.M.C.*

*** *Prepared at pH 7 0 from yolk sac cultures as described in Memo. No. 5 (5).*

during which period they were frequently agitated, the mixtures were stored at 4° C. overnight. On the following morning, the mixtures were centrifuged to remove the rickettsiae, and the sera were titrated for residual antibody. In each test, a similarly diluted control sample of serum was prepared and treated in the same way except that rickettsiae were not added. The chief relationships of the antigens and antibodies participating in the complement fixation reactions are shown in the following experiments. The first of these presents the results of cross absorption tests with the epidemic and murine strains, while the others provide information concerning the heat stability of the antigens involved in the complement fixation reactions.

Quantitative Cross Absorption Tests with Epidemic and Murine Strains

In these tests, different absorbing doses of rickettsiae were used for each serum. The results, which are summarized in Table I, show that rickettsiae of heterologous type can effectively absorb an antibody to an antigen common to both types but are unable to remove an antibody that reacts specifically with the homologous type. The homologous type, on the other hand, absorbs both antibodies with equal facility. Figs. 1 and 2 present the results of the quantitative absorption tests in graphic form.

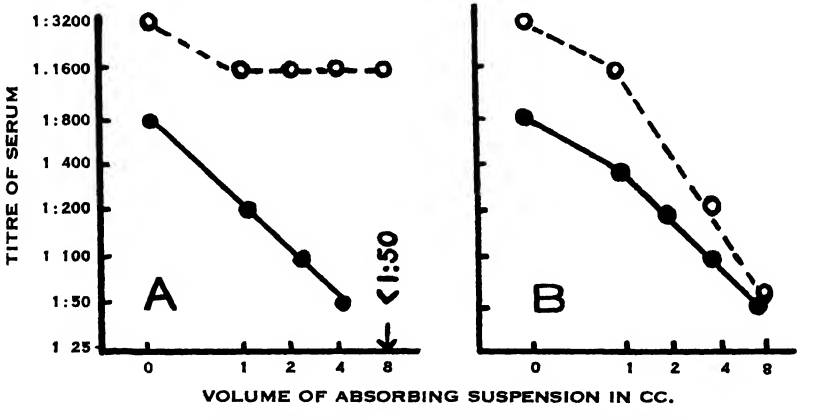

FIG. 1. *Quantitative absorption tests. A = Breinl convalescent serum absorbed with Breinl rickettsiae; B = Breinl convalescent serum absorbed with murine rickettsiae.* ● *Titre for Breinl antigen.* ○ *Titre for murine antigen.*

These tests (Table I) indicate that there are two type specific antigens and a common antigen and thus would seem to provide evidence against the view that the cross reactions between epidemic and murine strains result from the presence in these strains of two antigens, epidemic and murine, in different proportions. It may be noted that these tests also indicate that the specific complement-fixing antigens of the Breinl and Madrid strains are identical.

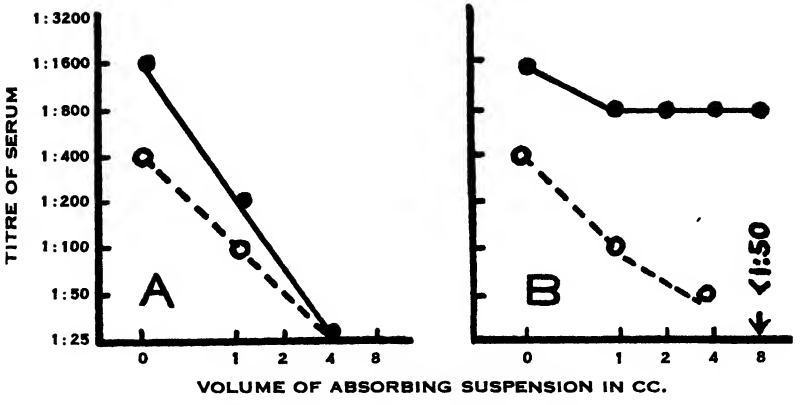

FIG. 2. *Quantitative absorption tests. A = murine convalescent serum absorbed with Breinl rickettsiae; B = murine convalescent serum absorbed with murine rickettsiae.* ● *Titre for Breinl antigen.* ○ *Titre for murine antigen.*

TABLE I

QUANTITATIVE CROSS ABSORPTION TESTS WITH EPIDEMIC AND MURINE TYPHUS RICKETTSIAE

Serum	Date of testing	Treatment of serum		Complement-fixing titre of serum		
				Breinl soluble antigen	Madrid soluble antigen	Murine soluble antigen
Breinl pooled convalescent guinea-pig serum	7/7/43	Not absorbed		1 : 1600	1 : 1600	1 : 400
		Absorbed with Breinl	1 cc.	1 : 200	1 : 200	1 : 100
			2.5 cc.	<1 : 100	--	<1 : 100
			4 cc.	1 : 25	1 : 25	1 : 25
		Absorbed with murine	1 cc.	1 : 800	—	1 : 100
			2.5 cc.	1 : 800	—	1 : 100
			4 cc.	1 : 800	1 : 800	1 : 50
			8 cc.*	1 : 800	—	<1 : 50
Murine pooled convalescent guinea-pig serum	7/7/43	Not absorbed		1 : 800	1 : 800	1 : 3200
		Absorbed with murine	1 cc.	1 : 400	—	1 : 1600
			2.5 cc.	1 : 200	—	—
			4 cc.	1 : 100	1 : 200	1 : 200
			8 cc.	1 : 50	—	1 : 50
		Absorbed with Breinl	1 cc.	1 : 200	—	1 : 1600
			2.5 cc.	1 : 100	—	1 : 1600
			4 cc.	1 : 50	1 : 100	1 : 1600
			8 cc.*	1 : 50	—	1 : 1600
Madrid pooled convalescent guinea-pig serum	10/8/43	Not absorbed		1 : 1600	1 : 3200	1 : 200
		Absorbed with Madrid	1 cc.	1 : 100	1 : 100	<1 : 50
			8 cc.	<1 : 50	<1 : 50	<1 : 50
		Absorbed with Breinl	1 cc.	1 : 800	1 : 800	1 : 100
			8 cc.	<1 : 50	1 : 50	<1 : 50
		Absorbed with murine	1 cc.	1 : 1600	1 : 1600	1 : 100
			4 cc.	1 : 1600	1 : 1600	1 : 50
			8 cc.*	1 : 1600	1 : 1600	<1 : 50

— *Not tested.*

* *Tests made 10/8/43.*

The Thermostability of the Complement-fixing Antigens

Tests have been carried out with antigens heated for periods of one hour in order to determine the temperature at which the specific complement-fixing antigen loses its ability to react *in vitro*. These have indicated that the critical temperature of inactivation of the specific antigen lies between 56° and 65° C. and that the residual antigen is shared by the epidemic and murine types.

For example, Breinl convalescent serum that had been absorbed with murine rickettsiae (Table II) fixed complement with unheated antigens (soluble antigen and also washed rickettsiae) although it failed to do so with the same antigens after they had been heated at 65° C. The unabsorbed serum contained an antibody that fixed complement with the heated antigens.

TABLE II

Effect of heat on the complement-fixing activity of the specific antigens of epidemic and murine rickettsiae

Test antigen	Date of testing	Treatment	Complement-fixing titre of Breinl convalescent serum	
			Not treated*	Absorbed with murine rickettsiae
Breinl soluble antigen	18/8/43	Untreated	1 in 1600	1 in 800
		Heated at 65° C.	1 in 400	<1 in 100
Breinl rickettsiae		Untreated	1 in 800	1 in 400
		Heated at 65° C.	1 in 200	<1 in 100
Murine soluble antigen		Untreated	1 in 400	<1 in 100

* *This serum contained, therefore, antibodies removed by the murine rickettsiae.*

It may, therefore, be concluded that the murine suspension that was used for absorption removed antibody that reacted with an antigen common to the Breinl and murine preparations, and that this common complement-fixing antigen was stable at 65° C. The tests with the absorbed serum, in which, presumably, only the specific antibodies remained, show that the specific complement-fixing antigen is labile at 65° C.

Table III shows the results obtained when a pooled Breinl convalescent serum was absorbed with heated Breinl rickettsiae. It will be noted that rickettsiae heated at 56° C. retained their capacity to absorb antibodies to heated and unheated soluble antigen but that rickettsiae heated at 65° C. failed to absorb antibody to the heat labile antigen.

II. The Specificity and Heat Lability of the Toxic Antigens of Epidemic and Murine Strains of Rickettsiae

When concentrated suspensions of infective typhus rickettsiae are inoculated intraperitoneally or intravenously into white mice, death occurs in one to eight hours (2, pp. 25–27; 9) Small doses of vaccine protect mice against this

TABLE III

ABSORPTION OF BREINL CONVALESCENT SERUM WITH UNHEATED AND HEATED BREINL RICKETTSIAE

Treatment of serum	Date of testing	Titre of serum for Breinl soluble antigen treated at the following temperatures		
		Not heated	56° C.	65° C.
Untreated	12/8/43	1 in 800	1 in 800	—
Absorbed with unheated rickettsiae		<1 in 50	<1 in 50	—
Absorbed with rickettsiae heated at 56° C.		<1 in 50	<1 in 50	—
Untreated	24/8/43	1 in 800	—	1 in 400
Absorbed with unheated rickettsiae		<1 in 50	—	<1 in 50
Absorbed with rickettsiae heated at 65° C.		1 : 800	—	<1 in 50

— *Not tested.*

toxic effect. This protection has been found to be highly specific when small doses of vaccine are used, although some cross protection is evident when a considerable multiple of the minimal immunizing dose is used (Memo. No. 4 (4)). A number of experiments have recently been undertaken to obtain further information concerning the specificity and heat stability of the antigens that stimulate immunity to the toxic factor of rickettsiae. Since the results have been consistent, only a few will be described.

The vaccines used were either samples of production lots of vaccine submitted for the routine mouse antigenicity test, or specially prepared small experimental lots. The technique of testing the potency of vaccines has been described in Memorandum No. 6, Part V (6) and need not be detailed here.

Table IV summarizes a comparison of the antigenicity of an experimental vaccine before and after heating at 50° C. and 56° C. for 45 min. The mice received a single dose of 0.5 cc. of diluted vaccine intraperitoneally. The vaccine was so diluted that 0.5 cc. contained 0.01 cc. of vaccine at standard concentration. Three weeks after vaccination, the mice were tested for immunity by intraperitoneal injection of a toxic preparation of rickettsiae.

It would appear from the results of a number of tests that 56° C. is the critical temperature at which inactivation of the specific immunizing antigen becomes apparent. Other tests have shown that vaccine may be kept at 42° C. for several weeks without a demonstrable loss of antigenicity.

Table V shows the results of cross protection tests with the Breinl and Madrid epidemic strains and the Castaneda murine strain. Vaccines heated at 56° C. were compared with untreated vaccine. Doses of vaccine ranging

TABLE IV

THE EFFECT OF HEAT ON THE IMMUNIZING VALUE OF TYPHUS VACCINE FOR MICE

Vaccine	Treatment of vaccine	No. of vaccinated mice surviving test with the following doses of yolk sac culture of Breinl strain		
		$\frac{1}{100}$th	$\frac{1}{200}$th	$\frac{1}{400}$th
56 *ES* 7/4	Not heated	8/8**	8/8	—
0.01 cc. at	50° C. for 45 min.	7/8	8/8	—
*S.C.**	56° C. for 45 min.	1/8	3/8	—
Controls	No vaccine	0/8	3/8	5/8

* *S.C.* = *standard concentration.*
** *Denominator* = *no. of mice tested.*
Numerator = *no. of mice surviving.*

TABLE V

CROSS PROTECTION TESTS IN MICE WITH EPIDEMIC AND MURINE VACCINES

Vaccine	Treatment of vaccine	Dose of vaccine at *S C* in cc	Number of mice surviving when tested with following toxic preparations								
			Breinl			Madrid			Murine		
			1/150	1/300	1/600	1/100	1/200	1/400	1/300	1/600	1/1200
Breinl lot	Not	0 3	4/4			4/4			1/4		
No 94	heated	0 1	4/4			4/4			4/4		
		0 03	4/4			3/4			3/4		
		0 01	4/4			3/4			1/4		
		0 003	3/4			3/4			2/4		
	56° C. for	0 3	2/4			4/4			3/4		
	45 min	0 1	4/4			3/4			3/4		
		0 03	3/4			4/4			0/4		
		0 01	2/4			1/4			1/4		
		0 003	0/4			0/4			2/4		
Madrid lot	Not	0 3	4/4			4/4			2/4		
No 78	heated	0 1	4/4			4/4			1/4		
		0 03	4/4			4/4			2/4		
		0 01	4/4			3/4			1/4		
		0 003	2/4			4/4			1/4		
	56° C for	0 3	1/4			0/4			0/4		
	45 min	0 1	0/4			0/4			2/4		
		0 03	2/4			0/4			0/4		
		0 01	0/4			0/4			1/4		
		0 003	0/4			0/4			2/4		
Murine lot	Not	0 3	3/4.			3/4			4/4		
No. 92	heated	0 1	3/4			2/4			4/4		
		0 03	2/4			3/4			4/4		
		0 01	0/4			1/4			4/4		
		0 003	1/4			0/4			4/4		
	56° C. for	0 3	2/4			1/4			0/4		
	45 min.	0 1	0/4			0/4			0/4		
		0 03	0/4			0/4			0/4		
		0 01	0/4			1/4			0/4		
		0 003	0/4			0/4			0/4		
Controls		None	0/8	2/8	5/8	0/8	2/8	6/8	0/8	4/8	5/8

Note: Breinl challenge—26/7/43; Madrid challenge—3/8/43; murine challenge—28/7/43.

from 0.003 cc. to 0.3 cc. at standard concentration were used in order to obtain quantitative data on the heat lability of the toxic antigen and on the relationship of the epidemic and murine antigens.

The results obtained with mice inoculated with unheated vaccine and tested with toxic preparations of the homologous strain of rickettsiae indicate a considerable degree of specific protection*. The cross tests with the Breinl and Madrid strains do not reveal any difference between these two epidemic types. In contrast to this specific protection, there is evidence of an irregular cross protection between the epidemic and murine strains. When vaccines in this experiment were given in doses 30 to 100 times greater than their minimal immunizing dose for the homologous type they protected only 40 to 75% of mice against the heterologous type.

The inactivation of the immunizing antigen at 56° C. is well shown in the case of the Madrid and murine vaccines. The Breinl vaccine on this occasion was not completely inactivated at 56° C., although the results show that significant reduction occurred. In this connection, the possible influence of traces of formalin on stability at the critical temperature should be borne in mind.

III. The Differentiation of Specific and Other Neutralizing Antibodies to Epidemic and Murine Strains of Typhus Rickettsiae by Means of the Giroud Rabbit Skin Reaction

Giroud (10) found that typhus immune serum neutralized the capacity of preparations of rickettsiae to produce a cutaneous reaction in rabbits. This cutaneous serum protection test has not given entirely satisfactory results in the hands of some investigators, but the possible advantages of a reliable skin test for rickettsial neutralizing antibodies prompted a further study. Encouraging results have been obtained by the introduction of certain modifications of the method. These modifications are based on the following observations.

There is a qualitative difference between the lesions produced (*a*) by a suspension of infective rickettsiae that has had its activity reduced by simple dilution and (*b*) the same suspension that has had its activity reduced as a result of exposure to immune serum. This difference is primarily one of ratio of the diameter to the thickness of the lesions. A partially neutralized mixture produces a lesion that is elevated, bright to dusky red in colour, with a clearly defined border. The diameter of the lesion is less than that of the lesion produced by the non-neutralized control mixture. Exactly similar lesions are not produced by higher dilutions of the rickettsial suspension in protective fluid; the redness and thickness of the lesion varies with the dilution of the material and with higher dilutions the border is poorly defined. If a

**Addendum, 2nd October, 1943.*

Since this paper was prepared, cross immunity tests have been carried out in which vaccinated mice were tested by intravenous injection of toxic preparations. The results confirm the specificity of immunity to the toxic factors of epidemic and murine strains and also the heat lability of the toxic antigen.

sufficient dose of infective virus, i.e., one that produces a well marked lesion, is used to test for neutralizing antibody, there is no difficulty in making accurate comparative measurements of the lesions produced by the control and immune serum mixtures.

A study of the quantitative relationships between the concentrations of virus and serum has shown that neutralization follows the percentage law first described by Andrewes and Elford in the case of bacteriophage neutralization and later shown to be operative in the neutralization of viruses (3). The appearance of complete neutralization can be obtained only with high titre sera and a relatively weak concentration of virus. Consequently, any method that attempts to titrate sera to a null end-point sacrifices sensitivity and consistency of results. More satisfactory results are obtained when the estimation of neutralizing antibody is based on the relative degree of reduction of the size of the lesion. Such estimation is possible because there is a relationship between the concentration of antibody and the dimensions of the lesion. This relationship will be explained after a brief description has been given of the procedure followed in carrying out the neutralization test.

Three fourfold dilutions are made of the serum to be tested. A standard concentration of a yolk sac culture of rickettsiae, which is used also as a reference control, is added to each dilution and the mixtures are shaken at intervals during a period of two hours at room temperature. Each mixture is then diluted 1 in 30, and 0 2 cc. volumes of the dilutions are injected intradermally. The maximum and minimum diameters of the lesions are measured at their height on the third or fourth day. In addition, the thickness of the lesion is estimated on a scale of 0, $\frac{1}{2}$, 2, 3, or 4. The thickness of the reference control lesions should have a value of 3 or 4.

Let us suppose then that A represents the cube root of the product of the three dimensions of the unneutralized control, and that X_1, X_2, and X_3, respectively, represent the cube roots of the products of the dimensions of each of the three lesions representing the three dilutions of immune serum.

If X_1, X_2, and X_3 are plotted as abscissa on a uniform scale against the corresponding dilution of serum as ordinate on a logarithmic scale, the three points will lie approximately on a straight line. The slope of this line is a constant for all sera tested together on one animal. This line is extrapolated to intersect a line drawn parallel with the ordinate from a point on the abscissa representing A, the size of the control lesion*. The point of intersection of these lines indicates the titre of the serum. A reference control serum is used in each group of tests because of variations in the skin sensitivity of rabbits. This use of a reference serum permits correction of the calculated titre of sera with the result that the titre can be determined with an accuracy that is within the limits of a twofold dilution.

Observations on many guinea-pig and human sera have shown that epidemic immune sera neutralize murine virus to a significant extent, and conversely,

* *The size of the control lesion is permitted to vary from 14 × 14 × 3 mm. to 17 × 17 × 4 mm. in different tests in different rabbits.*

that murine sera neutralize epidemic virus. The senior author had found that the typical rabbit skin reaction, which reaches its maximum in three to four days depends on the presence of infective rickettsiae and that high concentrations of killed rickettsiae are required to produce a transient skin reaction. This latter reaction reaches its height in 24 to 36 hr. and fades rapidly. The same author was also able to demonstrate that immune serum prevented this reaction. These observations suggested that neutralizing antibody inhibition tests with non-infective rickettsiae might indicate whether epidemic and murine immune sera contained two distinct neutralizing antibodies.

These inhibition tests were carried out with the following materials.

Sera.—These were obtained from guinea-pigs that had been injected with yolk sac cultures of the Breinl epidemic or the Castaneda murine strains. The bleedings were made 21 days after the onset of fever.

Inhibiting Suspensions of Rickettsiae.—The rickettsiae were separated by centrifugation from typhus vaccine processed as described in Memorandum No. 5 (5), suspended in saline and then purified further with ethyl ether. The concentration of rickettsiae in these repurified stocks was determined photometrically.

Infective Cultures of Rickettsiae Used to Test for Neutralizing Antibody.—These were yolk sac cultures, prepared and preserved in the same way as the toxic material used in the mouse test (6). The preparations were subjected to a preliminary titration to determine their optimum dilution for the estimation of neutralizing antibody. The Madrid No. IV strain was used in preference to the Breinl to test for epidemic type antibody since it produces a more marked and uniform reaction.

Two sets of inhibition tests were carried out, one with Breinl convalescent serum, the other with murine convalescent serum. In both sets of tests the following inhibiting suspensions were used.

(*a*) Breinl, not heated.
(*b*) Breinl, heated at 56° C. for 45 min.
(*c*) Murine, not heated.
(*d*) Murine, heated at 56° C. for 45 min.

Each suspension was mixed with 0.125 cc. of serum and saline was added to bring the volume to 1 cc. thus giving a 1 in 8 dilution of serum. The amounts of suspension that were used were such as to contain approximately 0.125 mgm. to 0.5 mgm. of rickettsiae (dry weight estimated from photometric data). After addition of the inhibiting suspension, the mixtures were shaken at intervals and allowed to stand overnight at 4° C. Thereafter, each mixture was divided into two parts. One of these was added to an infective yolk sac preparation of the Madrid strain and the other was added to a murine preparation. After a period of two hours at room temperature, during which the mixtures were shaken frequently, each mixture was diluted 1 in 30 and 0.2

cc. was injected intradermally. The resultant lesions were measured on the third and fourth days.

The results of the tests are presented in a simplified form in Figs. 3 and 4. The length of each column is the reciprocal of the size of the lesion produced.

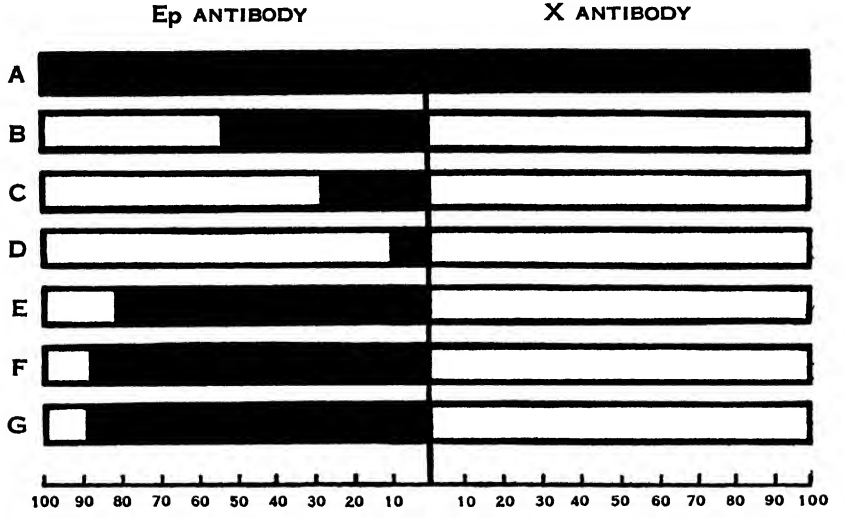

FIG. 3. *Neutralizing antibody inhibition tests; Breinl convalescent guinea-pig serum. Percentage of free neutralizing antibody remaining after addition of the inhibiting suspension is indicated in black.*

Ep—Antibody neutralizing the Madrid No. 4 strain.

X—Antibody neutralizing the murine strain.

A—Untreated serum control containing 225 units of Ep antibody and 55 units of X antibody.

B—Serum inhibited with 0 125 mgm. of Breinl suspension.

C—Serum inhibited with 0 25 mgm. of Breinl suspension.

D—Serum inhibited with 0 5 mgm. of Breinl suspension.

E—Serum inhibited with 0 125 mgm., 0 25 mgm., or 0.5 mgm. of Breinl suspension heated at 56° C.

F—Serum inhibited with 0.5 mgm. of murine suspension.

G—Serum inhibited with 0 5 mgm. of murine suspension, heated at 56° C.

Residual X antibody less than 10% in E and less than 5% in B, C, D, F, and G.

Since there is a quantitative relationship between the amount of neutralizing antibody and the reduction in size of the skin lesion, these reciprocals may, for the present purpose, be taken as equivalent to the percentage of antibody that was not inhibited. The titres of the uninhibited serum controls were as follows:

Breinl serum: 225 neutralizing units for Madrid virus, and 55 neutralizing units for murine virus.

Murine serum: 230 neutralizing units for murine virus, and 45 neutralizing units for Madrid virus.

The results are analogous to those reported for complement fixing antibodies in Section II of this paper. Inhibition with the heterologous type failed to

remove neutralizing antibody to the homologous type. Thus, it would appear that there are two neutralizing antibodies that participate in the Giroud cutaneous serum protection test, one representing a specific antigen, the other an antigen shared by epidemic and murine types. The inhibition tests with heated suspensions of rickettsiae show that the specific antigens are labile at 56° C. (Figs. 3 and 4).

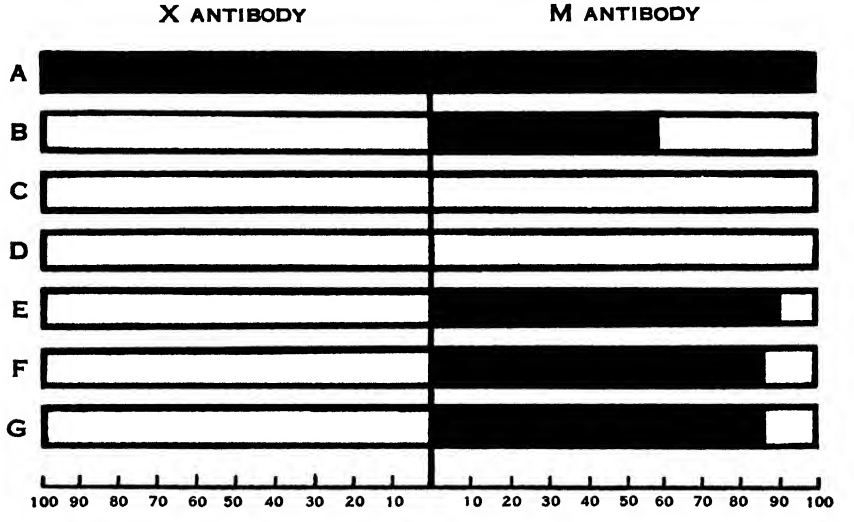

FIG. 4. *Neutralizing antibody inhibition tests, murine convalescent guinea-pig serum. Percentage of free neutralizing antibody remaining after addition of the inhibiting suspension is indicated in black.*

M— Antibody neutralizing the murine strain.

X—Antibody neutralizing the Breinl strain.

A—Untreated serum control containing 230 units of M antibody and 45 units of X antibody.

B—Serum inhibited with 0 125 mgm. of murine suspension.

C—Serum inhibited with 0 25 mgm. of murine suspension.

D—Serum inhibited with 0 5 mgm. of murine suspension.

E—Serum inhibited with 0 125 mgm., 0 25 mgm., or 0 5 mgm. of murine suspension heated at 56° C.

F—Serum inhibited with 0 5 mgm. of Breinl suspension.

G—Serum inhibited with 0 5 mgm. of murine suspension heated at 56° C.

Residual X antibody less than 5% in B, C, D, E, F, and G.

IV. The Neutralizing Antibody Response of Guinea-pigs Inoculated Intraperitoneally with Typhus Vaccine Heated at 60° C.

The results of some preliminary comparative tests of untreated and heated vaccine are of interest since they suggest a diversity of rickettsial antibodies and the need of further study to elucidate the relative importance of these antibodies in immunity.

The vaccine used in these tests was prepared from the yolk sacs of eggs inoculated on the sixth day of development with the equivalent of one-millionth of a yolk sac of Breinl culture. Eleven yolk sacs were collected

from the 10th to 12th day after inoculation and showed a particularly heavy infection. They were processed as described in Memorandum No. 5 (5). The vaccine thus obtained (56 *ES/D* 8/2(2)) showed the high dilution factor of 1 in 11.2. At this dilution, the vaccine represented less than a 1% suspension of yolk sac by weight. The preceding transfers of the Breinl strain in this series of egg passages had involved mouse blood and embryo blood transfers, and mouse antigenicity tests were therefore undertaken to determine whether any demonstrable antigenic change or degradation had occurred. The results of these antigenicity tests, carried out with 0.01 cc. of the vaccine diluted 1 in 11.2 to reduce it to standard concentration, provided further evidence that the immunizing value of vaccine prepared by the methods described in Memorandum No. 5 (5) is primarily related to the concentration of rickettsiae present.

At a later date, in connection with investigation of the heat lability of the mouse immunizing antigen, mice and guinea-pigs were vaccinated with this vaccine (56 *ES/D* 8 2(2)). The mice received the usual dose equivalent to 0.01 cc. at standard concentration. The guinea-pigs received 0.5 cc. of vaccine in terms of a 10% yolk sac suspension. The vaccines were heated in a water-bath regulated within 0.2° C. and were used on the same day. The intraperitoneal route of inoculation was used for all animals. Three weeks after vaccination, the mice were tested for active immunity and the guinea-pigs were bled out.

TABLE VI

VACCINE 56 *ES/D* 8/2(2)

MOUSE ANTIGENICITY TEST

Treatment of vaccine	Dose of vaccine at standard concentration	No. of mice surviving test with following doses of Breinl yolk sac		
		$\frac{1}{120}$	$\frac{1}{240}$	$\frac{1}{480}$
None	0.01 cc.	8/8	8/8	—
Heated at 56° C. for 45 min.	0.01 cc.	2/8	8/8	—
	0.03 cc.	3/8	8/8	—
	0.1 cc.	8/8	7/8	—
	0 3 cc.	8/8	8/8	—
Controls	No vaccine	0/8	1/8	4/8

Table VI indicates that a temperature of 56° C. for 45 min. reduced the antigenicity of the vaccine for mice by approximately 90%. Table VII*b* shows that heating at 60° C. for 45 min., which markedly reduced the mouse antigenicity (Table VII*a*), did not appreciably affect the ability of the vaccine to stimulate complement-fixing antibodies in tests that did not differentiate antibodies for the heat labile and heat stable antigens (Table VII*b*). In fact, the mean response to the heated vaccine was slightly greater than the

TABLE VII*a*

VACCINE 56 *ES/D* 8/2(2). Heated 7/5/43'

MOUSE ANTIGENICITY TEST

Treatment of vaccine	Dose of vaccine at standard concentration	No. of mice surviving test with following doses of Breinl yolk sac		
		$\frac{1}{250}$	$\frac{1}{500}$	$\frac{1}{1000}$
None	0 01 cc.	6/8	8/8	—
Heated at 60° C. for 45 min.	0.01 cc.	1/8	4/8	—
Controls	No vaccine	0/8	5/8	8/8

TABLE VII*b*

VACCINE 56 *ES/D* 8/2(2). HEATED 7/5/43

COMPLEMENT-FIXING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.* I.E. 0 5 CC. OF THE EQUIVALENT OF A 10% YOLK SAC SUSPENSION

Treatment of vaccine	Guinea-pig number	Complement-fixing titre of serum with following soluble antigens		
		Breinl	Madrid	Murine
None	2089	Not tested	—	—
	2090	Partial 1 in 100	Partial 1 in 100	Neg. 1 in 100
	2091	1 in 400	1 in 400	Neg. 1 in 100
	2092	Neg. 1 in 50	Neg. 1 in 50	Neg. 1 in 50
	2093	Not tested	—	—
	2094	1 in 800	1 in 800	1 in 200
Heated at 60° C. for 45 min.	2095	1 in 400	1 in 400	1 in 200
	2096	1 in 400	1 in 400	Partial 1 in 100
	2097	1 in 1600	1 in 1600	1 in 800
	2098	Neg. 1 in 100	Neg. 1 in 100	Neg. 1 in 100
	2099	Neg. 1 in 100	Neg. 1 in 100	Neg. 1 in 100
	2100	1 in 100	1 in 100	Partial 1 in 100

mean response to the untreated vaccine. The guinea-pig sera were pooled and tested for neutralizing antibody, by the modified Giroud test described in Section III, and also by the mouse method described by Henderson and Topping (12, pp. 41–56). The results of these tests are given in Table VII*c*.

Another set of tests was carried out on the same vaccine heated at 60° C. on another occasion (Tables VIII*a* and VIII*b*). Again, a lack of correlation between complement-fixing antibody and neutralizing antibody response was evident. As in the previous experiment, the heated vaccine stimulated neutralizing antibodies as shown by the Giroud test with the Madrid epidemic strain. Two pairs of sera, representing unheated and heated vaccines,

TABLE VII*c*

VACCINE 56 *ES/D* 8/2/(2). HEATED 7/5/43

NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY (SEE TABLE I)

MOUSE TEST 26/8/43

Treatment of vaccine	Pool guinea-pig sera	Neutralizing units by Giroud test with Madrid strain	Serum–virus mixture		No. of mice surviving intravenous inoculation of serum–virus mixtures	
			Dilution of serum	Fraction of Breinl yolk sac	For 6 hr.	For 24 hr.
None	2090		1 in 32	1/320	6/6	6/6
	2091	250	1 in 64	1/320	6/6	6/6
	2092		1 in 128	1/320	6/6	5/6
	2094		1 in 256	1/320	0/6	0/6
			1 in 512	1/320	0/6	0/6
Heated at 60° C. for 45 min.	2095		1 in 16	1/320	6/6	6/6
	2096		1 in 32	1/320	0/6	0/6
	2097	60	1 in 64	1/320	0/6	0/6
	2098		1 in 128	1/320	0/6	0/6
	2099		1 in 512	1/320	1/6	1/6
	2100					
Controls	Normal guinea-pig serum		1 in 16	1/320	0/6	0/6
			1 in 16	1/640	1/6	1/6
			1 in 16	1/1280	6/6	6/6

TABLE VIII*a*

VACCINE 56 *ES/D* 8/2(2). HEATED 3/5/43

COMPLEMENT-FIXING AND NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.*, I.E. 0 5 CC. OF THE EQUIVALENT OF A 10% YOLK SAC SUSPENSION

Treatment of vaccine	Guinea-pig No.	Neutralizing units by Giroud test with Madrid strain	Complement-fixing titre of serum with following soluble antigens		
			Breinl	Madrid	Murine
None	2070	Not tested	1 in 400	1 in 400	Neg. 1 in 100
	2071	Neg.	Neg. 1 in 125	Neg. 1 in 125	Neg. 1 in 125
	2072	42	1 in 200	1 in 200	1 in 100
	2074	110	1 in 200	1 in 100	Neg. 1 in 125
	2076	85	1 in 800	1 in 400	Neg. 1 in 100
	2077	8	1 in 200	1 in 400	Neg. 1 in 100
Heated at 60° C. for 45 min.	2078	Not tested	—	—	—
	2079	160	1 in 200	1 in 200	1 in 100
	2081	Not tested	1 in 200	1 in 200	1 in 100
	2082	25	1 in 400	1 in 400	1 in 200
	2083	17	1 in 100	1 in 200	Neg. 1 in 100
	2084	85	1 in 400	1 in 400	1 in 400

TABLE VIII*b*

VACCINE 56 *ES/D* 8/2(2). HEATED 3/5/43

NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.* (SEE TABLE II)

Treatment of vaccine	Pool of guinea-pig sera	Neutralizing units, by Giroud test with Madrid strain	Serum virus mixture		No. of mice surviving intravenous inoculation of serum virus mixture	
			Dilution of serum	Fraction of Breinl yolk sac	For 6 hr.	For 24 hr.
None	2074	110	1 in 32	1/320	6/6	6/6
	2076	85	1 in 64	1/320	6/6	6/6
			1 in 128	1/320	6/6	6/6
			1 in 256	1/320	1/6	1/6
			1 in 512	1/320	1/6	1/6
Heated at 60° C. for 45 min.	2079	160	1 in 8	1/320	6/6	6/6
	2084	85	1 in 16	1/320	6/6	6/6
			1 in 32	1/320	6/6	6/6
			1 in 64	1/320	0/6	0/6
			1 in 128	1/320	0/6	0/6
Controls	Normal serum	—	1 in 8	1/320	0/6	0/6
			1 in 8	1/640	0/6	0/6
			1 in 8	1/1280	6/6	6/6

respectively, were selected on the basis of the results of the Giroud test and pooled for mouse test. The results of the mouse neutralization test are given in Table VIII*b*. Although the sera from the guinea-pigs receiving heated vaccine had a neutralizing titre of 1 in 32, there was no correlation between the neutralizing titres determined by the rabbit skin test and the mouse. This, however, means very little since other observations (Section III) demonstrate the existence of two neutralizing antibodies participating in the the Giroud serum protection test and the consequent necessity of resorting to inhibition test to determine the titre of specific neutralizing antibody.

Discussion

The observations that have been described indicate that epidemic and murine typhus rickettsiae have the antigenic structures shown diagrammatically in Fig. 5. The evidence in support of this scheme may be summarized as follows, according to the kind of test used to differentiate the antibodies developed in response to the specific and common antigens.

Agglutination Tests

Earlier tests by the senior author in which use was made of agglutinating sera produced in the rabbit showed that absorption of Breinl sera with murine rickettsiae, and vice versa, resulted in removal of antibody responsible for cross agglutination reactions but that specific antibody remained. The antigen reacting with the specific antibody was found to be labile at 56° C.

A recent report (1) describes more extensive observations on a variety of strains, and the writers of this report conclude that both murine and epidemic rickettsiae possess a specific "species" antigen and share another antigen common to strains of the *OX*19 group.

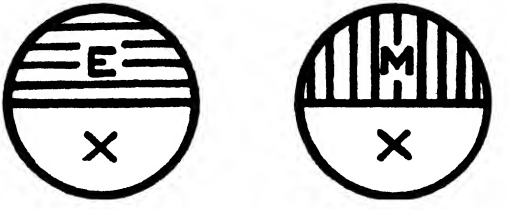

FIG. 5. *Antigenic structure of epidemic and murine typhus rickettsiae.*
E—heat labile specific epidemic antigen.
M—heat labile murine specific antigen.
X—heat stable common antigen.

Complement Fixation Tests

Absorption of convalescent guinea-pig serum with typhus rickettsiae of the type causing the infection removes antibodies to the homologous and heterologous types with equal facility. The amount of antibody removed is proportional to the absorbing dose. Absorption of the same serum with typhus rickettsiae of the heterologous type gives an entirely different result. In this instance, the antibody that reacts with the heterologous type is removed quantitatively in proportion to the absorbing dose, but antibody that reacts specifically with the homologous type is not removed. These effects are shown in Fig. 1. It is clearly evident that the Breinl serum contains antibodies that are not absorbed by the murine suspension. Similarly, the murine serum contains antibodies that are not absorbed by the Breinl suspension. There seems to be no alternative to the conclusion that epidemic and murine strains share a common complement-fixing antigen stable at 65° C. and that each possesses, in addition, its own specific heat labile antigen. The murine strain has no demonstrable trace of specific epidemic antigen and the converse applies to the epidemic strains.

The Giroud Test for Neutralizing Antibodies

As shown in Section III (Figs. 3 and 4) it is possible to demonstrate inhibition of neutralizing antibodies. The inhibition test developed for the differentiation of antibodies is the analogue of the absorption test as applied to agglutinating and complement-fixing antibodies. It differs from the absorption test in that it is unnecessary to remove inhibiting antigens from the serum.

Here again, the same antigenic pattern emerges (see Figs. 3 and 4) as in the case of the complement-fixing antigens. The antibodies measured in this case, however, are neutralizing antibodies. It follows, therefore, that the shared, or common, X antigen stimulates the development of antibodies that function as neutralizing antibodies when the Giroud rabbit cutaneous reaction

is employed to measure the percentage of rickettsiae surviving contact with the serum. Thus, the neutralizing titre of a whole serum, as measured by the rabbit skin test, merely indicates the sum or combined effect of two neutralizing antibodies that may be present in varying proportions in different sera.

The Mouse Test for Neutralizing Antibodies

Although no definite proof is available, it seems reasonable to assume that active immunity of mice to intraperitoneal inoculation of toxic preparations is predominantly humoral, because neutralization of the toxic factor *in vitro* can be so clearly demonstrated (12, pp. 41–56). Since the heterologous type of vaccine confers no regular cross immunity even when large doses are used in the mouse antigenicity test (Table V), it may be inferred that an antibody developed to the specific antigen is essential to complete immunity to the toxic factor. This does not mean, of course, that antibody to the common X antigen does not play an important role in active immunity. Perhaps both antigens are equally important.

Hamilton (11) has investigated the specificity of epidemic and murine rickettsial toxins and has concluded that the antibodies neutralizing these toxins are immunologically distinct. In the absorption tests reported by Hamilton, rickettsiae of heterologous type failed to remove the toxin-neutralizing antibody to the homologous type. He also noted that the neutralizing antibody was more readily absorbed by rickettsiae than were the complement-fixing antibodies.

It has been observed that vaccine heated at 60° C. in order to inactivate the mouse immunizing antigen, retained its ability to stimulate the development of neutralizing antibody in the guinea-pig (Tables VII*c* and VIII*b*). This stresses the need for further investigation of the complex antigenic structure of rickettsiae and the apparent multiplicity of corresponding antibodies. It should be noted that the guinea-pig sera used in the tests presented in Tables VII*b*, VII*c*, VIII*a*, VIII*b* were obtained by intraperitoneal, not subcutaneous, inoculation of heated vaccine. These sera appeared to neutralize the toxic factor for mice less effectively than they neutralized the skin-reacting factor (Tables VII*c*, VIII*b*). It will be necessary to resort to cross absorption tests before this difference in the results of the two neutralization tests can be satisfactorily explained.

Reference should be made to Felix's suggestion of an analogy between the heat labile and heat stable antigens of rickettsiae and the Vi and O antigens of *B. typhosum*. This analogy seems fully justified. In regard to pathogenic rickettsiae in general, Felix has expressed the opinion that further subdivision will be made possible by the study of the heat labile rickettsial antigens. Perhaps the analogy between the heat labile antigens of typhus rickettsiae and the Vi antigen of *B. typhosum* is more complete than Felix has ventured to suggest. The apparent differences between the heat lability of the specific mouse immunizing antigen (56° C.) and the specific complement fixing antigen

(60° C.), and the discrepant results of active immunity and neutralization tests (particularly the Giroud test) recall the "functional deficiency" of the Vi antigen described by Felix and Bhatnagar (8). Here, however, the analogy would appear to end because formalin seems to promote stability of the labile mouse immunizing antigen.

In conclusion, it should be pointed out that the observations reported here do not exclude the possibility that some strains of rickettsiae develop small amounts of the heat labile specific antigen that predominates in the heterologous type. It will be necessary to resort to cross absorption tests with heated rickettsiae and to an antigenic analysis of intermediate strains in order to obtain information about this interesting question. At least it can be said that the immunological reactions of the strains that have been examined are dominated by the two specific heat labile antigens and the heat stable X antigen.

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THE EFFECTS OF OESTROGENS AND OF MILD CHRONIC STARVATION ON THE WHITE RAT[1]

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Abstract

Daily injections of peanut oil for 17 or 18 days cause decrease in rate of growth of the rat, and testicular atrophy. Hence results in experiments in which peanut oil (and probably similar oils) are used as solvent vehicles for administration of material by injection may be misinterpreted. Oral administration of oestradiol (3 mgm. daily) to young mature rats for three weeks or more causes decrease in growth rate, relative decrease in size of kidneys, heart, spleen, muscle, and ovaries, little effect on the liver, marked decrease in size of testes, even to actual atrophy, and frequent enlargement of the adrenals in males, with occasional enlargement in females. The adrenals are discoloured to a maroon shade, whether enlarged or not, and evidence is advanced that they are undergoing a pathological change; any enlargement is not in the nature of hypertrophy. Oral administration of stilboestrol gives similar results. The general effects of oestrogens are more marked in male than in female animals. Loss of appetite and diminished food intake are among the general effects, but the mild chronic starvation so produced can only contribute in very minor degree to the other oestrogenic effects.

In mild chronic starvation from food restriction the liver is invariably affected, while the adrenals are never enlarged nor discoloured.

The effects of combined oestrogenic and thyroid administration seem to be neither additive nor truly antagonistic.

Introduction

Effects of Oestrogens

Published data show some lack of agreement, probably owing to such causes as method of dosage, size of dose, vehicle used for injection, variation in length of administration, variation in potency of the oestrogen used, and in age and strain of the test animal (cf. 15). The discrepancies in the published results are best revealed by considering separately the effects on growth rate, and on the different body organs. The rat is referred to, unless it is otherwise stated.

Growth rate is said to be slightly decreased by injected oestrone (8, 32), markedly decreased in young animals by oestradiol (25), and initially increased in mature females by oestradiol (23). It is decreased by stilboestrol given orally (21, 24), or by pellet implantation (22), or injected dissolved in sesame oil (20) or in corn oil (13). Stilboestrol administered orally to parent females

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depresses the growth rate of their suckling young, although growth rate is relatively less retarded in young than in mature animals (21). It is decreased to actual loss of weight by benzestrol, whether injected, given orally, or implanted in pellets (2).

The heart, kidneys, and liver are said to show a relative decrease in size in normal animals (25), and a relative or actual increase in size in gonadectomized animals (14) following treatment with oestradiol or other natural oestrogens. The spleen shows a relative decrease (25). Benzestrol is said to produce no appreciable effect on kidneys or liver (2).

The thyroid, following injections of oestradiol, is variously reported to be but little affected (25), decreased in size (7), and initially hypertrophied, then atrophied (23). Both natural oestrogens and stilboestrol are said to produce destruction of acinar walls (17). Benzestrol is said to produce no appreciable effect (2).

The ovaries are said to hypertrophy if sufficient oestrogen is injected (12) but most investigators claim that they atrophy (7, 20, 21, 22, 25, 23). Oestradiol and stilboestrol both produce atrophy of the testes (2, 25, 20, 21, 22).

(The islets of Langerhans in mice are said to enlarge following administration of oestrogen (9).)

The natural oestrogens produce enlargement of the pituitary (12, 15, 7, 23, 14, 26) except in immature rats; in these, such treatment tends to produce a decrease in size (26). Stilboestrol also produces an enlargement (20, 21, 22).

(Oestrogens are reported to cause reappearance of the *X* zone in the adrenals of mice (5), and, oppositely, to produce degenerative changes (9, 10).)

In rats, enlargement of the adrenals, usually considered to be a hypertrophy, is stated to be produced by the natural oestrogens (27, 12, 7, 25, 14, 3, 26), although some investigators report little or no effect (2, 15), and even decrease in weight (8, 26), final atrophy (23), and degeneration (11). Stilboestrol is said to cause enlargement of the rat adrenal (20, 21, 22, 13) and to produce haemorrhage into the cortex (24, 28, 16), and, on the other hand, to produce little or no effect (17); it causes enlargement of guinea-pig adrenals (1). Following administration of benzestrol there is a slight tendency to enlargement with occasional slight haemorrhage (2).

Some of the effects produced by stilboestrol have been shown to be proportional to dosage. By feeding it in drinking water in gradually increasing concentration it has been shown that growth is affected first, then the gonads (atrophy), while definitely higher dosage is needed to produce enlargement of the adrenals and pituitary (21).

In so far as it is possible to draw conclusions from majority verdicts in these discrepant reports, it seems that, for the normal rat, natural oestrogens cause decrease in growth rate, relative decrease in size of heart, kidneys, liver, and spleen, atrophy of ovaries and testes, and enlargement of the pituitary and adrenals, provided dosage be adequate. A relatively greater effect is produced on male than on female animals. Stilboestrol and benzestrol

produce effects comparable to those of natural oestrogens on growth rate, the gonads, the adrenals, and the pituitary.

It is generally agreed that many of the effects of the oestrogens are mediated through the pituitary. Adrenal enlargement (28, 27, 12, 30), depression of growth rate (24), and atrophy of the ovaries (12) are usually considered to be secondary effects of this kind, though there is evidence that body growth may be directly affected (21).

Effect of Vehicle Used to Dissolve Oestrogen for Purposes of Injection

Selye (25) injected cholesterol (presumably an inert compound) dissolved in peanut oil into young rats. His results suggest a slight decrease in weights of liver and ovaries in females, and of testes in males. These effects may well be due to the peanut oil.

Richards and Kueter (24) administered stilboestrol in oil, with resulting pronounced cloudy swelling in liver and kidneys; this effect was shown to be produced by prolonged administration of the oil alone.

Heskett and Huffman (13) injected stilboestrol in corn oil, and noted that, regardless of dosage, the animals became lethargic, their coats rough and coarse, their conjunctivae hyperaemic, and they developed a nasal discharge, diarrhoea, skin eruptions, etc., and lost appetite. The results were attributed to the stilboestrol, but not improbably were partly due to the vehicle.

Bruce and Tobin (4) record that daily injections of 0 25 to 1 .0 cc. of sesame oil for several weeks produce toxic effects on normal male rats, including inhibition of growth, decreased weight of testes, and increased adrenal weight.

Combined Oestrogen and Thyroid Administration

This has some practical significance, in view of the present minor vogue of treating hyperthyroid patients with oestrogens. It is well established that administration of thyroid to rats causes decreased growth rate, and hypertrophy of kidneys, heart, and adrenals, with lessened thyroid weight. Korenschevsky and Hall (14) consider that when both thyroid and oestrogen are administered they produce co-operative activity on adrenals, liver, kidneys, and heart, and a greater loss of body weight than is produced by either alone.

Claims have been made that administration of oestrogen to the experimentally hyperthyroid animal lowers oxygen consumption and decreases the basal metabolic rate (29).

Chronic Starvation

We are only concerned here with effects in the rat following chronic starvation for a period of some weeks. Mulinos and Pomerantz (18, 19) state that such chronic inanition produces atrophy of the adrenal cortex, never marked, and chiefly affecting the cytoplasm. The adrenal medulla is virtually unaffected. Loss of weight of the thyroid, spleen, and liver is relatively greater than that of the whole body. The weight and size of the ovaries are not markedly altered; anoestrus is usual.

Present Investigation

The experiments were begun in 1941 to ascertain whether the combined effects of oestradiol and thyroid administration were or were not additive. At first the oestradiol was injected dissolved in peanut oil, but since injections of peanut oil alone were found to produce definite effects, oestradiol was thereafter given orally. It seemed to produce lessened appetite, and the food intake was found to be measurably smaller. Hence the effect of mild chronic starvation was studied for comparison. Finally the effects of oestradiol and stilboestrol were compared, special attention being given to the adrenal glands.

Experimental Part

General

Each of many separate experiments were carried out with rats of the same sex and litter, each rat isolated in a separate cage. Individual variations of growth rates and of weights of organs of litter mates kept under precisely the same conditions have necessitated grouping the animals and averaging the results of groups. The muscle weighed for comparison in the experiments has been the right anterior tibialis.

Effect of Peanut Oil

When 0.1 cc. was injected intraperitoneally daily for 17 or 18 days, either alone or as vehicle for oestradiol, and then the rats were killed and their body cavities were opened up, the organ surfaces presented a peculiar glistening appearance due to a coating of minute oil droplets; this was especially noticeable on the surfaces of the liver, kidneys, and spleen.

In a few experiments the same dosage of peanut oil was injected alone and for the same periods as when used as solvent for oestradiol. The animals so treated showed a decrease in rate of growth and in weight of testes of the same order as that produced by the oestradiol solution. For example, the initial weight of a control male rat was 54.5 gm. It gained 49 gm. in 18 days and was then killed. Its testes weighed 1.33 gm. A litter mate initially weighed 56.5 gm. After 18 days' injection of 0.1 cc. peanut oil daily, it had only gained 31.5 gm., and its testes weighed only 0.38 gm.

It therefore seemed that results from experiments with oestradiol dissolved in peanut oil might be open to misinterpretation, and that conclusions as to the effect of oestradiol could be drawn more safely if it were given orally.

Experiments with Oestradiol, Given Orally

Table I gives the average results for groups of animals of the same sex and of similar initial body weights. In each single experiment the figures for the control, or the mean figures for controls, were taken for comparison. The effect on growth was calculated as the percentage ratio of the relative increases of body weight of the experimental and control animals during the experimental period. The effects on organ weights were calculated from the figures for percentage organ weights (i) based on body weights at the beginning

TABLE I

EFFECT OF OESTRADIOL ON THE GROWTH RATE AND BODY ORGANS OF THE WHITE RAT

	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
No. of treated rats	2	6	3	2	3	6	7	2
Length of treatment, days	15	21 (av)	21 (av)	31	15	23 (av)	22 (av.)	31
Average initial body wt., gm.	72	60	102	106	69	64	80	105
Average gain in wt., %	55	53	37	42	80	61	38	64
Organ wt. cpd. with normal, %								
Liver	96 (112)	106 (128)	90 (116)	87 (123)	110 (119)	100 (118)	103 (124)	99 (115)
Kidneys	91 (105)	97 (118)	88 (110)	78 (111)	101 (108)	96 (113)	87 (102)	80 (93)
Heart	87 (97)	96 (117)	82 (106)	76 (108)	97 (105)	92 (107)	91 (110)	86 (100)
Spleen	82 (96)	71 (85)	69 (85)	74 (103)	84 (91)	81 (95)	85 (106)	94 (111)
Muscle	76 (88)	91 (103)	78 (96)	69 (98)	87 (97)	90 (109)	—	91 (106)
Gonads	60 (69)	23 (27)	58 (72)	25 (35)	85 (91)	65 (76)	79 (93)	74 (86)
Adrenals	98 (112)	128 (155)	130 (165)	121 (173)	107 (116)	102 (118)	106 (132)	101 (117)
Thyroid	62 (74)	119 (144)	96 (121)	71 (100)	107 (115)	103 (119)	93 (113)	80 (88)

of the experimental period, and (ii) based on the final body weights. In Table I and subsequent tables the latter values are given in parentheses.

Throughout each experiment a daily dose of 3 mgm. crystalline oestradiol was fed each experimental animal. Attempts to give it with barley meal failed, but it was fairly well taken when mixed with carrot juice (controls got the carrot juice alone).

The effect on body organs can be analysed by applying the following criteria (remembering that values in parentheses are calculated from the percentage organ weights in terms of the final body weight):—

(*A*) When percentage organ weights, referred to initial body weight (i) are greater than those of controls (and thus exceed 100), actual enlargement is indicated; (ii) are equal to those of controls (giving a value approximately 100), there is no effect.

(*B*) When percentage organ weights are less than those of controls, when referred to initial body weight, but greater, when referred to final body weight, the effect on the organ is less than that of the whole body.

(*C*) When percentage organ weights, referred to final body weight, (i) are equal to those of controls (giving a figure in parentheses approximating to 100), the effect on the organ is proportional to that on the whole body; (ii) are less than those of controls, the effect is relatively greater than that on the whole body and may indicate an actual atrophy.

Examining Table I in light of these desiderata the following conclusions seem permissible. The figures show considerable variation (the variations for individual animals in a group were still greater). There is a greater effect

on males than on females. As might be expected there seems to be a more marked effect with longer treatment, though the number of animals in individual groups is too small to stress this.

Growth rate is invariably diminished.

Liver: there is no effect in females, a slight but negligible relative loss of weight in males.

Kidneys and heart: there is a relative decrease in size, but the effect is less than that on the whole body.

Thyroid: very variable results are shown; no definite conclusion can be drawn from them.

Spleen: in females it tends to remain proportional to the whole body, in males to be more markedly affected.

Muscle: in females it is less affected than the whole body; there is a somewhat greater effect in males.

Testes: marked relative decrease in weight, and sometimes actual atrophy.

Ovaries: the effect is greater than that on the whole body, but less than on the testes.

Adrenals: usually, but not invariably, there is an actual enlargement in male animals; there is usually but little effect in females, but sometimes some degree of enlargement.

Examination of individual records shows that when the body weight of male rats at the beginning of treatment was between 50 and 60 gm., oestradiol always produced an actual atrophy of the testes so that at the end of a 21-day experiment their combined weight varied from 0 16 to 0.25 gm.; with initial body weights between 60 and 80 gm., such atrophy was only occasionally produced, although there was always an arrest of growth. With still greater initial body weights, only some arrest of growth was obtained.

The colour of the adrenals in normal rats, in our experiments, has varied from bright yellow (rare) to dull and grey yellow, sometimes with a slightly reddish tinge. In practically all the animals fed (or injected) with oestradiol the adrenals showed a definite colour change, variously recorded as grey, brown, dark brown, and most frequently as brown red or maroon. Such change of colour was noted in enlarged glands, but also in others in which there was no definite enlargement, or even slight relative loss of weight.

Appetite

In association with the striking diminution in growth rate, it was observed that the rats fed oestradiol appeared to eat less food. Hence measurement of food intake was carried out in two experiments. Each day, weighed quantities of food were given in known excess of requirements, and the amount uneaten the following morning was removed, and weighed. Correction was made for partial drying of residues by several determinations of the water content of fresh material and of mixed residues. The correction is only

approximate, so that the measurements of food consumption also are only approximate. Nevertheless they should be comparable.

In Experiment 1, during the experimental period, each of a litter of four males and five females was fed daily 20 gm. bread and 25 gm. milk.

In 13 days the two control males ate respectively 446 and 465 gm. of the mixture (average 455.5 gm.). In the same period two males receiving 3 mgm. oestradiol daily ate 361 and 360 gm. (average 360.5); their intake was thus 21% less.

The corresponding figures for two control females were 369 and 431 gm. (average 400 gm.), and for the three females fed the same dose of oestradiol 429, 382, and 343 gm. (average 385 gm.); there was only a 4% diminished intake.

In Experiment 2 a litter of nine females was similarly treated. Four controls ate in 22 days 727, 733, 741, and 824 gm. (average 756 gm.). Five animals fed oestradiol ate 577, 625, 670, 726, 730 gm. (average 666 gm.), a diminished intake of 12%.

The apparently lesser effect on appetite of females than of males agrees with the other effects recorded.

The diminished food intake, although not great, suggests that some part of the effect produced by oestradiol might be traceable to a chronic mild starvation. Hence the effects of such a mild starvation were determined.

Effects of Chronic Mild Starvation

Five experiments were carried out in each of which animals on an insufficient diet were compared with controls of the same sex and litter. The controls were fed daily 20 gm. bread and 25 gm. milk; the experimental animals 8 gm. bread and 10 gm. milk. All residual food was removed each morning: the experimental animals left little. In a sixth experiment the daily allowance of the experimental animals was lowered to 4 gm. bread and 5 cc. milk and they actually lost weight, while becoming somewhat hyperactive. The results are given in Table II.

Analysis of the figures in Table II in accordance with the principles already stated permits the following conclusions. Liver, spleen, ovaries, and thyroid show a relative lessened gain of weight greater than that of the whole body. Kidneys, heart, testes, and muscle show a relative lessened gain of weight less than, or proportional to that of the whole body. Adrenals show a lessened gain of weight relatively less than that of the whole body in males, a somewhat greater effect in females.

In chronic starvation of the duration and degree of severity tested, enlargement of adrenals never occurred while the glands always retained their normal colour.

It is to be noted that the effect on growth rate was greater than that produced by oestradiol, in our experiments, so that the effects on body organs are as great or greater than can be attributed to the potential mild starvation

TABLE II

SUMMATION OF EFFECTS OF CHRONIC STARVATION ON GROWTH AND BODY ORGANS

	Males		Females	
	Group 1	Group 2	Group 1	Group 2
Type of starvation	Mild	Mild	Mild	Severe
No. of treated rats	3	7	5	2
Duration of expt., days	21	20-21	21	20
Average initial body wt., gm.	71	84	70	76.5
Relative increase in body wt. as cpd. with controls, %	26	19	35	−80
Organ weights cpd. with normal, %				
Liver	60 (97)	58 (88)	69 (89)	34 (97)
Kidneys	66 (107)	68 (104)	72 (95)	51 (143)
Heart	64 (106)	67 (104)	76 (100)	47 (134)
Spleen	55 (89)	58 (89)	72 (96)	31 (83)
Muscle	73 (122)	81 (124)	88 (113)	29 (79)
Gonads	82 (133)	74 (116)	61 (80)	23 (64)
Adrenals	80 (127)	84 (127)	67 (90)	41 (114)
Thyroid	52 (83)	58 (89)	59 (77)	47 (130)

of the oestradiol-fed rat. A comparison of the two sets of results indicates several definite differences. The liver is practially unaffected by oestradiol, but is very susceptible to inadequacy of food intake. Oestradiol has a marked effect on the testes, mild starvation but a slight effect. Chronic starvation never produces adrenal enlargement or colour change; oestradiol frequently produces enlargement, and usually produces colour change. Hence it seemed reasonable to conclude that the general effects produced by oestradiol are primary effects and are not appreciably affected by the loss of appetite it induces.

Effects of Stilboestrol

Pure crystalline diethylstilboestrol was used, and fed in varying doses in different experiments. Some difficulty was experienced in feeding it. It was not well taken in carrot juice, but was eaten moderately well in carrot pulp, or by alternately mixing it with carrot pulp and with bread and milk (controls being of course fed the medium alone). The results are summed up in Table III.

Comparison of Tables I and III suggests general similarity in the effects of oestradiol and stilboestrol. As with oestradiol, male animals appear to be more markedly affected than females. Markedly increased dosage does not appear to produce marked difference in results, but the number of animals in each group is too few to stress this, especially since there was not complete control of the actual amounts eaten in these oral experiments.

Stilboestrol rarely produced enlargement of the adrenals, though the same maroon discoloration was produced as with oestradiol. The apparent enlargement of the livers of female rats was probably fortuitous. The uteri of the stilboestrol animals were markedly distended (with a clear fluid); those of the oestradiol-fed animals were less affected.

TABLE III

EFFECTS OF STILBOESTROL ON GROWTH, ETC.

	Males		Females		
	Group 1	Group 2	Group 3	Group 4	Group 5
No. of test animals	4	3	4	4	3
Daily dosage, mgm.	3	6	2	3	6
Duration of expt., days	30–35	31	22	30–35	31
Average initial body weight, gm.	111 5	109	114	101	99 5
Relative increase in body wt. as cpd. with controls, %	27	36	29	49	54
Organ wt. cpd. with normal, %					
Liver	92 (140)	93 (130)	104 (122)	120 (140)	108 (128)
Kidneys	82 (118)	77 (110)	84 (99)	96 (113)	92 (109)
Heart	84 (119)	81 (113)	98 (118)	96 (113)	90 (110)
Spleen	73 (105)	66 (92)	82 (97)	77 (90)	79 (96)
Muscle	71 (102)	72 (102)	84 (103)	89 (95)	85 (100)
Gonads	17 (24)	18 (25)	114 (137)	89 (100)	94 (111)
Adrenals	108 (154)	95 (133)	77 (91)	102 (119)	83 (98)
Thyroid	66 (94)	50 (72)	76 (90)	101 (119)	66 (76)

Effect of Oestrogens on the Adrenals

An attempt was made in some of the later experiments to ascertain the nature of the adrenal enlargement and change in colour by determination of water content. No difference in colour as produced by either of the oestrogens could be definitely asserted, though the adrenals of the oestradiol-fed animals were (perhaps imagined to be) a little greyer.

The adrenals were transferred to closed weighing bottles as rapidly as possible after dissection, weighed, and then dried at 105° C. for 24 hr. The results are shown in Table IV.

The results suggest that there is a tendency to increased water content, whether or not there is an actual enlargement of the glands.

Since it has previously been shown that, in acute starvation, enlargement of the adrenals is in the nature of a hydropic degeneration, with increased water content, and decreased number of cells in corresponding areas of the adrenal cortex (6), an attempt was made to estimate the cell numbers in corresponding areas of the adrenal cortexes of animals fed oestrogens. The technique has already been fully described (6). The cell counts were made by counting nuclei in five 1 × 10 cm. strips running inward from the periphery of the cortex in a series of photomicrographs (magnification × 200), of which three or four were made for each adrenal, selecting central sections. The figures refer to results per square cm. of the measured areas. Data are given in Table V and are for a single litter of rats.

The results in Table V indicate that the slight enlargement of the adrenals produced in a male rat by stilboestrol was accompanied by a decreased number of cells in the outer part of the cortex. Examination of the photomicrographs also indicated a definite narrowing of the glomerular zone. The relative

TABLE IV

EFFECT OF OESTROGENS ON WATER CONTENT OF ADRENALS

Sex	Controls			Stilboestrol-fed					Oestradiol-fed				
	No. of animals	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.	
		Extremes	Mean				Extremes	Mean				Extremes	Mean
		%	%	Mgm			%	%	Mgm			%	%
Male	5	28–32	30	3	2	Enlarged	27–28	27 5	—	—		—	—
				3	1	?		26	—			—	—
				6	2	Relatively smaller	29–30	29 5	3	2	Enlarged	25–25	25
Female	7	27–32	30	2	4	Relatively smaller	26–29	27	—			—	—
				3	3	Relatively smaller	25–28	26	—			—	—
				6	1	Relatively smaller		21	—			—	—

TABLE V

CELL COUNTS IN COMPARABLE AREAS OF ADRENAL CORTEXES

	Rat 1, male	Rat 2, male	Rat 3, female	Rat 4, female	Rat 5, female	Rat 6, female	Rat 7, female
	Control	Stilboestrol	Control	Oestradiol	Oestradiol	Stilboestrol	Stilboestrol
Daily dose, mgm.	—	6	—	3	3	6	6
Initial body wt., gm.	109	123	86	110.5	105 5	105 5	99 5
Gain in body wt. (31 days), gm.	54	18	33	20 5	21 5	19	18
Left adrenal							
Actual wt., mgm.	13 5	19 4	24 7	30 6	27 4	19 8	23 6
Relative wt , mgm. %*	12 4	15 8	28 7	27 8	26.0	18 8	23 7
Cell count	63 2	42 6	26 1	27 3	26 0	29 9	28 0
Right adrenal							
Actual wt., mgm.	13 4	12 3	18 4	26.0	25.2	21.2	15 6
Relative wt., mgm. %*	12 3	10.0	21 4	23 5	23 9	20 1	15 7
Cell count	56 0	44 9	30 5	30.9	27 4	29 6	27.6
Both adrenals							
Actual wt., mgm.	26 9	31 7	43 1	56 6	52 6	41 0	39.2
Relative wt., mgm. %*	24 7	25 8	50.1	51 2	49 9	38 9	39.4
Cell count	59.6	43.7	28 3	29 1	26 7	29 7	27 8
Mean values				27 9		28.7	

To initial body weight.

decrease in weight of the adrenals of the stilboestrol-fed female rats was not accompanied by a change in cell count. There was an occasional thinning of the glomerular zone. In female animals fed oestradiol it produced no change in the adrenal weights. There was no change in cell count, but there was a marked decrease in the width of the glomerular layer.

Effect of Combined Oral Administration of Oestradiol and Desiccated Thyroid

A few experiments were carried out to ascertain this effect. In each, animals of the same sex and litter were fed daily either (i) desiccated sheep thyroid (0.32% iodine) in daily dosage of 1 : 5000 of the (initial) body weight, or (ii) 3 mgm. oestradiol, or (iii) a combined dose of the two. One or more animals (also of this litter and sex) were used as controls.

The results were not clear cut. They were certainly not additive. Thus, since both oestradiol and thyroid feeding depress growth rate, jointly they should give a greater depression, but this was not the case. Since (at least in male animals) both tend to produce adrenal enlargement, jointly they should produce greater enlargement, but they did not do so. They both depress the relative weight of the muscle tested, and jointly should do so to a greater extent, but did not do so. They both depress the development of the testes, but jointly produced no greater effect than oestradiol alone.

Nor did the results indicate a definite antagonism between the actions of the two, except perhaps on the heart; in both sexes, instead of the enlargement produced by thyroid feeding, following the combined treatment the weight of the heart remained practically normal.

The data obtained were so indefinite that it did not seem worthwhile to carry out further experiments.

Discussion of Results

General

In determining the effects of any particular treatment on growth or on one or several of the body organs of the rat it is usually considered adequate to take a number of animals of the same sex and of approximately the same initial body weight, and to divide them into two groups, one to be treated, the other kept as controls. In recorded experiments the size of such groups varies considerably. Careful examination of our records of control animals made during the past 25 years shows very marked variations both for rate of growth and for the organ weights at any particular body weight, so that large groups of randomly selected animals are essential to permit accurate conclusions concerning qualitative differences in results, unless the observed differences are constant and large. This is still more true where attempts are made to draw conclusions as to quantitative differences.

It is generally assumed that the degree of normal variation in litter mates of the same sex is much smaller than in a random selection. Our records show that this is usually the case, and that in plotted results, lines connecting values of litter mates for a particular organ (heart, kidneys, adrenals, etc.), tend to

parallel the average curve for such an organ, so that the percentage values tend to be in good agreement. Yet there are by no means infrequent exceptions, in which the reverse of such parallelism is seen, so that even with comparisons with litter mates, the average of a number of comparisons is desirable.

In some of the experiments now recorded it is recognized that the number of animals used has not been adequate; the results in such cases have not been stressed.

Peanut Oil

The definite effects on rate of growth and on the testes following injection of peanut oil indicate that there may be danger in drawing conclusions from experiments in which this oil is used as solvent vehicle for test material. It has been pointed out that Bruce and Tobin (4) obtained very similar results with sesame oil, while some of the pathological effects reported by Heskett and Huffman (13) may well be due to injected corn oil. It seems likely that the inertness of such oils has been unwarrantedly assumed. While the amounts employed therapeutically are almost certainly without significance, those used in experiments with small animals are relatively much greater and their potential effect should not be ignored.

The Effects of Administered Oestrogens

Oestradiol, in the dosage used for the periods of time stated, produced in our experiments results almost identical with the majority verdicts set out in the introduction. Effect on liver, however, seems negligible, and actual atrophy of ovaries seems doubtful, while enlargement of the adrenals was rare in female animals. There was definitely a greater effect on males than on females. Stilboestrol produced similar effects, our results in general agreeing with those of Noble (21), who administered it in drinking water.

The Effect of Oestrogens on the Adrenals

The enlargement has usually been considered a hypertrophy. We have shown that the so-called adrenal hypertrophy produced by acute starvation is in reality a pathological process in the nature of a hydropic degeneration, and we have adduced evidence from the literature that the similar enlargement that results from thiamin deficiency is also not a hypertrophy. We have stressed that whenever experimental treatment results in marked discoloration of the adrenals, this indicates presence of a pathological process (6).

We have now shown that such discoloration follows treatment with oestrogen (oestradiol or stilboestrol), the typical colour being a maroon or grey-maroon, and differing from the dark grey or dirty grey colour typical of the adrenal of the moribund acutely starved rat. This maroon discoloration occurs whether or not the adrenal is enlarged, or is even relatively somewhat smaller than normal.

We have also adduced some evidence suggesting a tendency to increased water content, not definitely associated with enlargement. Examination of histological sections of such glands suggested presence of no marked abnor-

mality such as haemorrhage, but there was a tendency to thinning of the glomerular zone, not definitely associated with enlargement. Cell counts in comparable areas of the cortex were normal in absence of enlargement, and decreased when enlargement had occurred.

Too few determinations and examinations were made to stress these findings, but they all fit together to permit the suggestion that oestrogens produce no hypertrophy of the adrenals, but rather, in the dosage used, something in the nature of a pathological process.

Mild Chronic Starvation

Our results are in moderate agreement with those of Mulinos and Pomerantz (18, 19), which were of longer duration. The absence of enlargement of the adrenals is to be stressed. The effects produced by oestrogens include loss of appetite and diminished food intake but the mild chronic starvation resulting can only influence the oestrogen results in very minor degree.

Combined Oestrogen and Thyroid Administration

Results in the few experiments performed were not in good agreement with those of Korenschevsky and Hall (14) and indicated neither an additive effect, nor a true antagonism.

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EFFECT OF REDUCING AGENTS ON THE VIABILITY OF EQUINE ENCEPHALOMYELITIS VIRUS (EASTERN TYPE)[1]

By N. A. Labzoffsky[2]

Abstract

The present communication deals with the effect of reducing agents (cysteine hydrochloride, sodium thioglycollate, and sodium formaldehyde sulphoxylate) on the viability of equine encephalomyelitis virus (Eastern type). Cysteine hydrochloride was found to be a valuable reagent in *in vitro* studies of equine encephalomyelitis virus, because it greatly retards loss of infectivity of the virus under experimental conditions. It was observed that a virus suspension containing cysteine hydrochloride (1 : 1000) remained infective after exposure to 37° C. for 14 days, although the guinea-pig titre was reduced from $1 : 10^8$ to $1 : 10^2$. The same reduction in the titre of virus suspended in buffered 0 85% sodium chloride solution occurred after exposure to 37° C. for 120 hr. only, and in unbuffered 0 85% sodium chloride after 24 hr. exposure. Further, equine encephalomyelitis virus, in the presence of cysteine hydrochloride, retains its infectivity without demonstrable loss, over a pH range between 4 8 and 8 2 for 48 hr. at 37° C. The titre of equine encephalomyelitis virus is maintained at 37° C. for 48 hr. in a rather wide range of Eh, created with the aid of cysteine hydrochloride, at least in the range between −0 151 and + 0 02 volts. On the other hand, addition of sodium formaldehyde sulphoxylate or sodium thioglycollate to a suspension of equine encephalomyelitis virus does not retard loss of infectivity of the virus. These reagents, therefore, are not suitable for the conservation of infectivity of the virus *in vitro*.

Introduction

The virus of equine encephalomyelitis, although relatively stable if kept frozen or stored in glycerol, loses infectivity quite readily, especially in high dilutions, when exposed to 37° C. or room temperature. This lability of the virus presented great difficulties in studying certain aspects of the neutralization reaction because of non-specific inactivation of the virus due to environmental factors (6). The most commonly employed diluting fluids, such as hormone broth, solutions consisting of a combination of several salts buffered to the required pH, or simple buffered physiologic solution of sodium chloride, containing 5 to 10% normal serum, although having a definite advantage over previously employed 0 85% solution of sodium chloride still provide only a moderate protection, insufficient for prolonged survival of the virus under experimental conditions. The environmental factors determining the viability of the virus *in vitro* under experimental conditions, in spite of extensive investigations, have not as yet been determined for viruses in general and no satisfactory preserving fluid has been developed. The most extensively investigated environmental factor is the pH and the stability of most of the viruses to various hydrogen ion concentrations has been definitely determined. Attempts have also been made to determine the effect of reducing agents on the viability of some of the viruses, but this question

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has not received sufficient attention from the investigators and the available information is limited and inconclusive. In this connection, Mueller (8) demonstrated that loss of infectivity of Rous chicken sarcoma virus due to oxidation upon exposure to 37° C. is deferred for more than 24 hr. and occasionally longer by addition of cysteine hydrochloride 1 : 2000. Similar results were obtained by Zinsser and Tang (13) with herpes virus. Schultz, Gebhardt, and Bullock (10) likewise found that cysteine exercises a beneficial effect on the viability of poliomyelitis virus. The results obtained by McKinnon (7) of the use of cysteine in connection with vaccinia virus are rather inconclusive. His experiments indicate that cysteine offered some slight protection when virus was stored at 2° and 5° C. and at 18° and 22° C. but at the temperature of 37° C. cysteine appeared to be detrimental to the virus. From McKinnon's presentation it appears that the experiments were conducted under aerobic conditions. Bang and Herriott (1) report of successful use of 0 1*M* cysteine for preservation of infectivity of equine encephalomyelitis virus during purification process.

The preliminary experiments conducted by the writer, using cysteine hydrochloride as a reducing agent, yielded highly encouraging results. It was found that equine encephalomyelitis virus diluted 1 : 100 would survive in the presence of 1 : 1000 cysteine hydrochloride, when exposed to 37° C. from 24 to 48 hr., without diminution in the titre, and up to two weeks with only a gradual decrease in the titre. When stored at 4° C. the above preparation has displayed a remarkable stability. It was found that after three months at refrigeration temperature there was no reduction in the titre, whereas in the control preparation (virus in buffered saline) no live virus could be detected. Even after 4½ years at 4° C. the preparation remained infective, although the titre dropped, as determined by guinea-pig inoculation, from $1 : 10^8$ to $1 : 10^4$, indicating approximately 0.1% survival. With these observations as a background, a more extensive investigation of the effect of cysteine on the viability of equine encephalomyelitis virus was planned. The possibility of developing suitable conditions for prolonged survival of the virus at 37° C. has been the subject of this investigation.

Plan of Present Investigation

With the view of determining favourable conditions for prolonged survival of equine encephalomyelitis virus under experimental conditions, the effect of reducing agents (cysteine hydrochloride, sodium thioglycollate, and sodium formaldehyde sulphoxylate) on the viability of the virus was investigated. The problem was approached from the following angles:

1. The rate of loss of infectivity of the virus particles at 37° C. suspended in buffered saline containing cysteine hydrochloride was compared with that of the virus suspended in diluents without cysteine hydrochloride.

2. An attempt was made to determine the optimal range of reducing intensity for the viability of the virus at optimum pH.

3. Investigation was made of the stability of the virus at 37° C. under conditions of optimum Eh and varying pH.

4. The effects of two other reducing agents, namely, sodium thioglycollate and sodium formaldehyde sulphoxylate were investigated for comparison.

Materials and Methods

The Virus

Equine encephalomyelitis virus, Eastern type, isolated by Schofield and Labzoffsky (9), from the blood of an infected horse at St. George, Ont., during the 1938 epizootic, was employed in this investigation. The virus has been propagated, since its primary isolation, alternately in guinea-pigs and in developing chick embryos.

The infected chick embryos, usually sixth or seventh egg passage, were harvested from the 16th to 18th hour after inoculation, when moribund or dead, then macerated, and without preliminary dilution the material was distributed in 4- to 5-cc. volumes into a number of test-tubes, plugged with cotton and stored in the freezing chamber of an electric refrigerator. A portion always was reserved for bacterial cultures. Prior to any one of the tests, one of the tubes was removed from the refrigerator, thawed at room temperature, and then centrifuged in a Swedish angle centrifuge at 4000 r.p.m. for 20 min. The supernatant fluid was collected, a portion was removed for immediate animal titration and sterility test, the rest of the fluid was frozen until the results of animal titration and sterility test were available. The end point of the virus was found to be very consistent, never being less than $1 : 10^8$ and occasionally $1 : 10^9$.

The Normal Serum

The normal serum, which gave negative neutralization reaction with the above virus, was pooled serum from a number of normal guinea-pigs of varying weights. The serum was inactivated at 58° C. for 45 min.

Diluent

The diluent was 0.85% sodium chloride either unbuffered or buffered at the required pH. The final concentration of phosphate buffer used was 0.004 *M.*

Reducing Agents

The reducing agents used were cysteine hydrochloride, supplied by Eastman Kodak Co., sodium thioglycollate, and sodium formaldehyde sulphoxylate produced by the Baltimore Biological Laboratories.

The reducing agents, for use in the experiments, were prepared as follows: 0.1 gm. of reducing agent was dissolved in 15 cc. of double distilled water, filtered through a Seitz E.K. pad previously washed with distilled water. The final volume was carefully measured and adjusted to the required pH.

For adjusting pH of a reducing agent either sodium monohydrogen phosphate or sodium dihydrogen phosphate of 0 1 *M* was used, depending on the reducing agent employed. Cysteine hydrochloride, being strongly acid, was adjusted with sodium monohydrogen phosphate, while for adjusting the pH of sodium thioglycollate and sodium formaldehyde sulphoxylate, sodium dihydrogen phosphate was used. After the pH of the reagent was adjusted the further dilutions were made in buffered saline. The volume of the reagent used for the adjustment of the pH was taken into consideration when the final concentration of the reducing agent was considered.

Test Mixtures

A given volume of the dilution of the virus in buffered saline containing 10% normal guinea-pig serum was mixed with an equal quantity of the reducing agent of desired concentration. As soon as the preparation of the mixture was completed, a sample was set aside for immediate potentiometric and animal titrations and the rest was distributed into 1 cc. ampoules with capillary necks, using a syringe and needle, then sealed and placed in an incubator at 37° C. for the required length of time. As a control the parent concentration of the virus was diluted 1 in 2 with either buffered or unbuffered physiologic salt solution, as required, so that it contained the same concentration of the virus as the test mixtures. This was also sealed in ampoules and incubated at 37° C. After the exposure of the mixtures to 37° C. for a given time, potentiometric and animal titrations of the mixtures were made. For the titration of the residual virus 10-fold dilutions of the mixtures were made in buffered physiologic saline containing 5% normal guinea-pig serum. A fresh pipette was used for making each dilution. Inoculations were made as soon as the dilutions were completed, starting with the highest dilution. The same syringe and needle were used for each series of increasing concentrations.

Test Animals

Healthy guinea-pigs weighing about 400 gm., previously used for tetanus antitoxin toxicity tests, were employed for the titration of the mixtures. All inoculations were made intracerebrally, without anaesthesia, the dose being 0 2 cc. Never fewer than two guinea-pigs were used for each dilution.

Potentiometer

All potentiometric determinations were made with Leeds and Northrop Student's Potentiometer. The glass electrode was used for measuring pH and gold for Eh measurements. Before each use, the potentiometer was checked with standard buffered solutions. Not more than five Eh determinations were made before recleaning the gold electrode with chromic–sulphuric-acid cleaning mixture.

Further details of the methods will receive mention in the description of individual experiments.

Experimental Data

Effect of Cysteine Hydrochloride on the Survival of the Virus of Equine Encephalomyelitis at 37° C.

In this experiment the comparative death rate of equine encephalomyelitis virus at 37° C. in buffered, in unbuffered physiologic solution of sodium chloride, and in buffered salt solution containing cysteine hydrochloride was investigated. Three preparations were made:

(1) Virus diluted 1 : 10^3 in unbuffered physiologic solution of sodium chloride containing normal guinea-pig serum 1 : 20.

(2) Virus diluted 1 : 10^3 in buffered physiologic solution of sodium chloride containing normal guinea-pig serum 1 : 20.

(3) Virus diluted 1 : 10^3 in buffered physiologic solution of sodium chloride, containing cysteine hydrochloride 1 : 1000 and normal guinea-pig serum 1 : 20.

A portion of each of the preparations was reserved for immediate animal titration and the remainder was distributed in a number of 1 cc. ampoules, sealed, and placed in the incubator at 37° C. At different intervals the mixtures were titrated on guinea-pigs for the residual virus. Usually a pool of not fewer than three ampoules of the same preparation was used for the titration. Three guinea-pigs were used for each dilution The time of exposure to 37° C. and the results of animal titration are shown in Table I. No potentiometric titrations were made; the pH, however, was checked colorimetrically before and after the exposure to 37° C.

It will be seen from the table that cysteine, in the concentration used, has definitely exercised a beneficial effect on the viability of the virus. The rate of deterioration of the virus has been considerably retarded and even after 14 days of exposure to 37° C. live virus has been recovered from the initial concentration. The deterioration of the virus became demonstrable after 48 hr. at 37° C. and reached apparent equilibrium after 96 hr. of exposure. This equilibrium lasted through to the sixth day, after which the loss of infectivity of the virus particles became more accelerated. Deterioration of the virus in the buffered saline, on the other hand, was considerably faster. The titre of the virus after 48 hr. at 37° C. dropped from 1 : 10^8 to 1 : 10^6, indicating approximately 1% of survival. After 120 hr. of exposure approximately only 0.001% of the virus survived and no live virus was detected after that. In unbuffered saline, only approximately 0 0001% survived 24 hr. exposure to 37° C. and no live virus was demonstrable in the preparations exposed for 48 hr.

The results obtained in this experiment were highly encouraging and it became clearly desirable to extend this work in order to obtain more complete data on the influence of cysteine hydrochloride in maintaining the viability of the virus. For this reason in the next experiment an attempt was made to determine the effect of different reducing intensities on the viability of the virus.

TABLE I

COMPARATIVE EFFECT OF DIFFERENT DILUENTS ON THE VIABILITY OF EQUINE ENCEPHALOMYELITIS VIRUS AT 37° C.

Dilution of virus inoculated	Virus diluted 1 in 100 in:																			
	0 85% NaCl			Buffered 0 85% NaCl						Buffered 0 85% NaCl and cysteine 1 : 1000										
	Time at 37° C.																			
	0 hr.	24 hr.	48 hr.	0 hr	48 hr.	72 hr	96 hr.	120 hr.	6 days	0 hr	48 hr.	72 hr	96 hr.	120 hr.	6 days	7 days	8 days	9 days	11 days	14 days
$1 : 10^2$	—	3,4	S,S	—	—	—	3,3,3	3,3,S	S,S,S	—	—	—	—	—	—	3,3,3	3,3,3	3,3,3	3,3,3	3,3,S
$1 : 10^3$	—	S,S	S,S	—	—	3,3,4	3,3,S	3,S,S	S,S,S	—	—	—	—	—	—	3,3,3	3,4,S	3,S,S	S,S,S	S,S,S
$1 : 10^4$	—	S,S	—	—	3,3,3	3,3,3	3,3,4	S,S,S	—	—	3,3,3	3,3,4	3,3,3	3,3,3	3,3,4	3,3,3	S,S,S	S,S,S	S,S,S	—
$1 : 10^5$	—	S,S	—	—	3,3,4	3,4,S	S,S,S	S,S,S	—	—	3,3,4	3,3,3	3,3,3	3,3,4	3,3,3	3,4,S	S,S,S	—	—	—
$1 : 10^6$	3,3	S,S	—	3,3	3,3,3	S,S,S	S,S,S	—	—	3,3	3,3,4	3,3,3	3,4,S	3,3,3	3,3,4	—	—	—	—	—
$1 : 10^7$	3,3	S,S	—	3,3	S,S,S	S,S,S	—	—	—	3,3	3,3,3	3,3,4	S,S,S	S,S,S	S,S,S	—	—	—	—	—
$1 : 10^8$	3,3	S,S	—	3,4	S,S,S	—	—	—	—	3,3	3,3,3	3,S,S	S,S,S	—	—	—	—	—	—	—
$1 : 10^9$	S,S	—	—	S,S	—	—	—	—	—	S,S	—	—	—	—	—	—	—	—	—	—
% Survival	100	0.0001	0	100	1 0	0 1	0 01	0 001	0	100	100	10	1 0	1 0	1 0	1 0	0 001	0 0001	0 0001	0 0001

Note: *Number = day of death.*
S = animal survived.
— = dilution not tested.

Effect of Varying Reducing Intensities, Created by Different Concentrations of Cysteine Hydrochloride, at Constant pH on the Viability of Equine Encephalomyelitis Virus

In this experiment, using cysteine hydrochloride as a reducing agent, the effect of different reducing intensities on the survival of the virus at 37° C. was investigated and an attempt was made to determine an approximate Eh range for the viability of the virus. The fact that the reduction of cysteine is not truly reversible, and that the system does not fall readily into line with classical examples of reversible oxidation–reduction reactions was borne in mind. Variation of reducing intensity in different preparations in this experiment was accomplished by using different concentrations of cysteine hydrochloride, while constancy of pH in the preparations was controlled by phosphate buffer.

Three concentrations of cysteine hydrochloride, adjusted to approximately pH 6.9, were made, namely 1 : 500, 1 : 3000, and 1 : 4000. The virus was diluted 1 : 500 in buffered physiologic solution of sodium chloride of pH 6.9 and containing 10% normal guinea-pig serum. Then equal volume of the above diluted virus was mixed with equal quantity of each of the above concentrations of cysteine. As a control the parent concentration of the virus was diluted 1 in 2 with buffered physiologic saline. Thus the resulting four preparations contained final concentration of virus, $1 : 10^3$; normal serum, 1 : 20; cysteine: preparation No. 1, 1 : 1000; preparation No. 2, 1 : 6000; preparation No. 3, 1 : 8000; and preparation No. 4 (control) none. The final pH of the preparations was approximately 6 9.

A sample was withdrawn from each mixture for the immediate potentiometric and animal titrations and the remainder of each mixture was sealed in ampoules and placed in an incubator at 37° C. for 120 hr., after which time the potentiometric and the animal titrations were carried out. The results are recorded in Tables II and III.

The Eh of the preparations prior to the exposure to 37° C., as is shown in Table II, were −0.151, −0.0022, and + 0 02 volts. After 120 hr. at 37° C.

TABLE II

Determination of potentials of virus preparations with constant pH and varying Eh before and after exposure to 37° C. for 120 hr.

Preparation	Concentration of virus	Concentration of cysteine	Prior to exposure to 37° C.		After exposure to 37° C. for 120 hr.	
			pH	Eh in volts	pH	Eh in volts
1	1 : 1000	1 : 1000	6.9	− 0 151	6 8	+ 0 0088
2	1 : 1000	1 : 6000	6 95	− 0 0022	6 85	+ 0 0337
3	1 : 1000	1 : 8000	6 9	+ 0 02	6 9	+ 0 0349
4	1 : 1000	—	6.9	—	6 85	—

TABLE III

ANIMAL TITRATION OF VIRUS PREPARATIONS WITH CONSTANT pH AND VARYING Eh BEFORE AND AFTER EXPOSURE TO 37° C. FOR 120 HR.

Dilution of virus in inoculum	Tested immediately				Tested after 120 hr. at 37° C.			
	Concentration of virus 1 : 1000							
	Concentration of cysteine			Control without cysteine	Concentration of cysteine			Control without cysteine
	1 : 1000	1 : 6000	1 : 8000		1 : 1000	1 : 6000	1 : 8000	
$1 : 10^3$	—	—	—	—	3,3,4	3,3,3	3,3,4	S,S,4
$1 : 10^4$	—	—	—	—	3,3,3	3,3,4	3,3,3	S,S,S
$1 : 10^5$	—	—	—	—	3,3,4	3,3,3	3,3,3	S,S,S
$1 : 10^6$	3,3	3,3	3,4	3,4	4,S,S	S,S,S	S,S,S	S,S,S
$1 : 10^7$	3,3	3,4	3,3	3,3	S,S,S	S,S,S	S,S,S	S,S,S
$1 : 10^8$	3,4	3,3	3,3	3,3	S,S,S	S,S,S	S,S,S	—
$1 : 10^9$	S,S	S,S	S,S	S,S	—	—	—	—

Note: *Number* = *day of death.*
S = *animal survived.*
— = *dilution not tested.*

the Eh of the mixtures has changed to + 0 0088, + 0 0337, and +0 0349 volts, respectively, while the pH was apparently unaffected or only slightly so.

The titre of the virus, as determined by guinea-pig inoculation, Table III, was the same in all three preparations containing cysteine hydrochloride, indicating that approximately the same percentage, namely 1%, of the virus survived in each of the preparations. In buffered saline control mixture no live virus was detected.

The results thus would indicate that the viability of the virus of encephalomyelitis is maintained in a rather wide range of Eh, at least in the range between −0 151 and + 0 02 volts, providing the pH of the preserving fluid is kept around 7. The results also provide further proof of the beneficial influence of cysteine in maintaining the viability of the virus. This becomes especially impressive when high initial dilution of the virus is taken into consideration and the fact that no live virus was detected in the control preparation.

In the next experiment the effect of different pH values on the survival of the virus in the presence of cysteine hydrochloride is investigated.

EFFECT OF CYSTEINE HYDROCHLORIDE ON THE STABILITY OF INFECTIVITY OF EQUINE ENCEPHALOMYELITIS VIRUS AT VARIOUS HYDROGEN ION CONCENTRATIONS

Effect of pH on the viability of different viruses under experimental conditions has been extensively investigated during recent years. The results of the detailed study of this question in case of plant viruses (11, 12) and some of the animal viruses (2, 3) indicate that for each individual virus studied there is a definite pH zone in which the stability of a given virus is most

pronounced. On either side of this zone there is a relatively rapid drop in stability. The virus of equine encephalomyelitis (Eastern type), however, as reported by Finkelstein and his co-workers (4, 5) appears to behave in a different manner. Their results show that at 0° C. to 5° C. the greatest stability range of infectivity of the above virus lay between pH 7.5 and pH 8.5. A second range of relative stability was found to be between pH 3.5 and pH 5.0. Between these two extreme ranges there is a region of rapid inactivation between pH 5.2 and pH 5.8, the extreme being at pH 5.5. From a practical standpoint it was felt desirable to determine whether or not this stability of equine encephalomyelitis virus in relation to pH is in any way influenced by the addition of cysteine hydrochloride. The pH range chosen for this study covers three points relative to the stability of the virus, namely, pH 4 8 (point of relative stability), pH 5 6 (point in a region of rapid inactivation), and pH 8 2 (point in the region of maximum stability).

Three buffer–virus mixtures with above pH values were prepared. Each mixture contained final concentration of virus $1 : 10^4$ and normal guinea-pig serum 1 : 20. In order to have the initial Eh approximately the same in each of the three mixtures, the concentration of cysteine hydrochloride had to be varied, thus Mixture I of pH 4.8 had concentration of cysteine 1 : 600, giving Eh of the mixture −0 1690 volts. Mixture II of pH 5.6 contained cysteine 1 : 800 and had Eh −0.1685 volts. Mixture III of pH 8.2 contained cysteine 1 : 1200, and had Eh −0 1705 volts. As a control three buffer–virus mixtures with corresponding pH values were prepared.

The results of the potentiometric and animal titration of the mixtures before and after the exposure to 37° C. for 48 hr. are given in Tables IV and V.

TABLE IV

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS WITH CONSTANT Eh AND VARYING pH VALUES BEFORE AND AFTER EXPOSURE TO 37° C. FOR 48 HR.

Virus preparation	Concentration of virus	Concentration of cysteine	Prior to exposure to 37° C.		After 48 hr. at 37° C.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 10,000	1 : 600	4 8	−0 1690	4 85	−0 0675
II	1 : 10,000	1 : 800	5 6	−0 1685	5 75	−0 045
III	1 : 10,000	1 : 1200	8 2	−0 1705	8 25	−0 0811
IV	1 : 10,000	—	4 8	—	4 75	—
V	1 : 10,000	—	5 6	—	5 8	—
VI	1 : 10,000	—	8 2	—	8.3	—

It will be seen from Table IV that the pH of the mixtures was practically unaffected by the exposure to 37° C., the slight difference recorded might have been due to a technical inaccuracy during the determination. Eh of the mixtures, on the other hand, has changed considerably, especially in Mixture II. The final Eh of the mixtures, however, remained on the negative side.

TABLE V

COMPARATIVE TITRE OF THE VIRUS IN THE PREPARATIONS WITH CONSTANT Eh AND VARYING pH VALUES AFTER EXPOSURE TO 37° C. FOR 48 HR., AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 10,000					
		Preparations containing cysteine			Control virus preparation in buffered saline		
		I 1 : 600 pH 4 85	II 1 : 800 pH 5 75	III 1 : 1200 pH 8 25	IV pH 4 75	V pH 5 8	VI pH 8 3
1 : 1	$1 : 10^4$	3,3,3	3,3,4	3,3,4	3,4,S	5,S,S	3,3,4
1 : 10	$1 : 10^5$	3,3,3	3,3,3	3,3,4	S,S,S	S,S,S	3,4,4
$1 : 10^2$	$1 : 10^6$	3,3,4	3,3,4	3,3,3	S,S,S	S,S,S	S,S,S
$1 : 10^3$	$1 : 10^7$	3,4,4	3,3,4	3,3,4	S,S,S	S,S,S	S,S,S
$1 : 10^4$	$1 : 10^8$	3,3,4	3,4,4	3,3,4	—	—	—
$1 : 10^5$	$1 : 10^9$	S,S,S	S,S,S	S,S,S	—	—	—
Initial titre of the preparations prior to exposure to 37° C. as determined by guinea-pig inoculation		$1 : 10^8$	$1 : 10^8$	$1 : 10^8$	$1 : 10^8$	$1 : 10^8$	$1 : 10^8$

Note: *Number* = *day of death.*
S = *animal survived.*
— = *dilution not tested.*

This change of Eh apparently had no detrimental effect on the viability of the virus as was shown by animal titration of the residual virus. The guinea-pig titre of all three mixtures after exposure to 37° C. for 48 hr. was found to be the same, $1 : 10^8$, indicating that approximately the same percentage of the virus has survived in each case. The titre of the control mixtures, however, has dropped considerably in comparison to the test preparations. This is especially pronounced in Mixture V with initial pH 5 6 where only one animal out of three inoculated with the highest concentration of the virus succumbed. In Control Mixture IV of pH 4 75 approximately 0.01% of the virus survived and in Mixture VI of pH 8.2 the approximate survival of the virus was 0.1%, that is 10 times as much as in Mixture IV. The results of the control mixtures are in close agreement with those obtained by Finkelstein and his co-workers (4, 5). On the whole the data obtained would indicate that addition of cysteine hydrochloride to the virus suspension minimized the effect on the virus of pH in the range studied. The virus appears to survive equally well for at least 48 hr. at 37° C. in pH range between 4.8 and 8.2. This fact may prove to be of significance when effect of the pH on the neutralization reaction is determined and also in connection with purification of the virus.

In the succeeding section the effect of other reducing agents on the virus is reported.

Effect of Sodium Thioglycollate and Sodium Formaldehyde Sulphoxylate on the Viability of Equine Encephalomyelitis Virus

In this series of experiments the effect of sodium thioglycollate and sodium formaldehyde sulphoxylate on the virus in question is investigated. Both these substances are now extensively employed in connection with cultivation of anaerobic bacteria and it was thought desirable to determine whether or not these reagents could be employed in the preservation of the virus. With this in view several different concentrations of these two substances were tested. Two experiments were conducted employing sodium formaldehyde sulphoxylate and one with sodium thioglycollate.

The final concentration of virus in all three experiments was $1 : 10^3$. The final concentration of sodium formaldehyde sulphoxylate in the first experiment was 1 : 800 and 1 : 3000; in the second 1 : 2500, 1 : 5000, and 1 : 10,000. The concentrations of sodium thioglycollate tested were 1 : 400 and 1 : 800. As a control, a suspension of virus of the same concentration in buffered saline was used. In the first experiment with sodium formaldehyde sulphoxylate the mixtures were exposed to 37° C. for 96 hr.; in the second and in the experiment with sodium thioglycollate the exposure was 72 hr. The results of potentiometric and animal titrations both before and after the exposure to 37° C. are reported in Tables VI, VII, VIII, IX, X, and XI.

In the first experiment with sodium formaldehyde sulphoxylate, Tables VI and VII, no live virus was detected, after the exposure to 37° C. for 96 hr., in the preparation containing the above reagent 1 : 800 nor in the control preparation. In the preparation containing the reagent 1 : 3000 only one guinea-pig out of two came down with encephalomyelitis in the dilution $1 : 10^4$. The results thus may indicate that some slight protection was offered by the above substance in the concentration of 1 : 3000. In the second experiment the titre of the virus, as determined by guinea-pig inoculation, in all three test mixtures containing different concentrations of sodium formaldehyde sulphoxylate, namely, 1 : 2500, 1 : 5000, and 1 : 10,000, was the same and somewhat higher than in the control, indicating some slight protection. The protective effect, however, was not as striking as that with cysteine hydrochloride. The results of the second experiment might be somewhat misleading and more striking difference between the titres of the test and control preparations might have been obtained if the time of the exposure to 37° C. was increased beyond 72 hr.

The results of the experiment with sodium thioglycollate appear to indicate that this reagent, besides having a low reducing intensity, is detrimental to the virus in a concentration of 1 : 400. The survival of the virus in the preparation containing the above reagent 1 : 800, Tables X and XI, taking into consideration the control preparation, may be attributed to the presence of buffer rather than to the effect of sodium thioglycollate. Neither of these reagents, therefore, compare favourably with cysteine hydrochloride.

TABLE VI

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING DIFFERENT CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 96 HR.

Preparation	Sodium formaldehyde sulphoxylate	Prior to exposure to 37° C.		After 96 hr. at 37° C.	
		pH	Eh in volts	pH	Eh in volts
I	1 : 800	7 5	− 0 1575	7 85	− 0.0742
II	1 : 3000	7 5	+ 0 0075	7 79	+ 0.0252
III	None	7 5	—	7 2	—

TABLE VII

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING DIFFERENT CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 96 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Tested immediately			Tested after exposure to 37° C. for 96 hr.		
		Initial concentration of virus 1 : 1000 in:					
		Sodium formaldehyde sulphoxylate		Buffered saline	Sodium formaldehyde sulphoxylate		Buffered saline
		1 : 800	1 : 3000	—	1 : 800	1 : 3000	—
1 : 1	$1 : 10^3$	—	—	—	S,S,S	3,3,3	S,S,S
1 : 10	$1 : 10^4$	—	—	—	S,S,S	4,S,S	S,S,S
$1 : 10^2$	$1 : 10^5$	—	—	—	S,S,S	S,S,S	S,S,S
$1 : 10^3$	$1 : 10^6$	3,3	3,4	3,3	S,S,S	S,S,S	S,S,S
$1 : 10^4$	$1 : 10^7$	3,4	3,3	3,3	S,S,S	S,S,S	S,S,S
$1 : 10^5$	$1 : 10^8$	3,3	3,3	3,3	—	—	—
$1 : 10^6$	$1 : 10^9$	S,S	S,S	S,S	—	—	—

Note: *Number* = *day of death.*
S = *animal survived.*
— = *dilution not tested.*

TABLE VIII

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR.

Preparation	Concentration of virus	Sodium formaldehyde sulphoxylate	Prior to exposure to 37° C.		After exposure to 37° C. for 72 hr.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 1000	1 : 2500	7.5	+ 0 004	7.3	+ 0 0021
II	1 : 1000	1 : 5000	7 5	+ 0.02	7.7	+ 0.033
III	1 : 1000	1 : 10,000	7.5	+ 0 0343	7.2	+ 0 041
IV	1 : 1000	—	7.5	—	7.4	—

TABLE IX

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 1000 in:							
		Sodium formaldehyde sulphoxylate						Buffered saline	
		1 2500		1 5000		1 . 10,000			
		Tested							
		Immediately	72 hr at 37° C	Immediately	72 hr at 37° C	Immediately	72 hr. at 37° C.	Immediately	72 hr at 37° C.
1 : 1	1 10^{3}	—	3,3	—	3,3	—	3,3	—	3,3
1 : 10	1 : 10^{4}	—	3,3	—	3,3	—	3,3	—	3,3
1 : 10^{2}	1 10^{5}	—	3,4	—	3,3	—	3,4	—	3,4
1 : 10^{3}	1 · 10^{6}	3,4	3,3	3,3	3,3	3,3	3,3	3,3	S,S
1 . 10^{4}	1 10^{7}	3,3	S,S	3,4	3,3	3,3	S,S	3,4	S,S
1 : 10^{5}	1 : 10^{8}	3,4	S,S	3,3	3,4	3,4	S,S	3,3	S,S
1 · 10^{6}	1 10^{9}	S,S	—	S,S	—	S,S	—	S,S	—

Note: Number = day of death.
S = animal survived.
— = dilution not tested.

TABLE X

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM THIOGLYCOLLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR.

Preparation	Concentration of virus	Sodium thioglycollate	Prior to exposure to 37° C.		After exposure to 37° C. for 72 hr.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 1000	1 : 400	7 5	+ 0 032	7 65	+ 0 0435
II	1 : 1000	1 : 800	7 5	+ 0 0345	7 75	+ 0.0485
III	1 : 1000	—	7 5	—	7.45	—

Discussion

Aside from providing evidence for the beneficial influence of cysteine hydrochloride on the viability of the virus of equine encephalomyelitis (Eastern type) the results reported raise problems concerning the electrode potential of the virus suspensions.

The beneficial effect of the addition of cysteine to the virus suspensions has been demonstrated quite conclusively, as the rate of loss of infectivity of the virus in the surroundings of high reducing intensity was considerably retarded. It also has been shown that the virus will survive in a rather wide

TABLE XI

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM THIOGLYCOLLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 1000 in:					
		Sodium thioglycollate				Buffered saline	
		1:400		1:800			
		Tested					
		Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.
1 : 1	1 : 10^3	—	S,S	—	3,3	—	3,3
1 : 10	1 : 10^4	—	S,S	—	3,4	—	3,3
1 : 10^2	1 : 10^5	—	S,S	—	S,S	—	S,S
1 : 10^3	1 : 10^6	3,3	S,S	3,3	S,S	3,3	S,S
1 : 10^4	1 : 10^7	3,4	S,S	3,3	S,S	3,4	S,S
1 : 10^5	1 : 10^8	3,4	—	3,4	—	3,3	—
1 : 10^6	1 : 10^9	S,S	—	S,S	—	S,S	—

Note: *Number* = *day of death.*
S = *animal survived.*
— = *dilution not tested.*

range of electrode potential, at least between −0 151 and + 0 2 volts. The findings regarding the viability of the virus at different H-ion concentrations, when Eh was kept at a low level, is of some practical interest. Although the pH range studied was rather limited, yet it serves to indicate the possibility of the virus surviving at even greater pH range to be of practical value in connection with purification and concentration of the virus. The presence of cysteine in the virus suspension appears to minimize the effect of pH to a great extent, since it was found that the virus survived equally well in the pH range between 4.8 and 8.2. The electrode potential of the virus suspensions was found to be always higher after prolonged exposure to 37° C. and no evidence of static equilibrium of the potential was observed in any of the experiments. Whether this rise of the potential was actually associated with the deterioration of the virus remains to be determined. Therefore, from the present data it is not at all clear what relation the potential of the virus suspensions measured bears to conditions within the living virus particle. This is obviously one of the fundamental problems.

The two other reducing agents, sodium thioglycollate and sodium formaldehyde sulphoxylate, employed in this study did not prove to be adequate for the purpose of preservation of the virus. Sodium formaldehyde sulphoxylate gives some slight protection, but hardly sufficient to be of practical value. Sodium thioglycollate, on the other hand, offers no protection, in fact, at least in the concentration of 1 : 400, it appears to be detrimental to the virus.

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THE *Rh* TYPES IN CANADIANS OF JAPANESE RACE[1]

BY B. CHOWN[2], Y. OKAMURA[3], AND R. F. PETERSON[4]

Abstract

The theory of multiple allelic genes accounting for the inheritance of the *Rh* reactions is well established. Fisher's hypothesis of eight genes, six pairs of allelic antigens, and six specific antibodies is the most comprehensive offered.

The blood of 606 Canadians of Japanese race was tested with the four available antisera. Since this sample included entire families, gene analysis was based on a subsample of 217 unrelated individuals. The resulting analysis, based on Fisher's hypothesis, is given, and the probable reason for a discrepancy in the findings presented and further tested. Estimates of gene frequencies in per cent were as follows:

R_1, 58.00; R_2, 30.76; R'' 2.89; R_z, 0.40; r, 7.95; R', 0; R_o, 0; R_y, 0.

These results are compared with previous studies on Japanese, and examined in relation to Fisher's hypothesis of three linked loci, and Fisher and Race's hypothesis of crossing over of elementary antigens.

Introduction

The theory of multiple allelic genes accounting for the inheritance of *Rh* reactions was developed independently in the United States and England (7 (p. 509), 8, 9, 12-18, 20-30). In January 1944, when six allelic *Rh* genes had been isolated, a probable seventh indicated, and four types of anti-*Rh* antibodies were known, Fisher (14) developed a comprehensive hypothesis postulating eight allelic genes, three pairs of allelic antigens, and six antibodies. Each gene, he suggested, was associated with the selection of three antigens from the three pairs, and every antigen could give rise to a specific antibody. The three pairs of antigens he denoted C, c, D, d, E, e, and the corresponding antibodies with which they reacted Γ, γ, Δ, δ, H, η. The relationships of the genes, antigens, and antibodies according to this hypothesis are set out in Table I. In it we have omitted the *h* from *Rh* in designating the genes, as is now common practice with British authors, and have given the antibodies or antisera the Greek letter designation of Fisher (14), the *Rh-Hr* designation of Wiener (24), and the English letter designations of Cappell (2), which we use in this paper. The one gene, R_y (not yet isolated), its assumed serological reactions, and the assumed reactions of the not-yet-discovered antibody anti-*d* are shown in parentheses.

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TABLE I

THE *Rh* SERIES OF ALLELIC GENES, ANTIGENS, AND ANTIBODIES MODIFIED FROM FISHER (14), WIENER (24), AND CAPPELL (2)

Gene	Combinations of elementary antigens	Antibodies and their reaction with antigens					
		Rh$_o$* Δ** *D****	*Rh'* Γ *C*	*Rh''* H *E*	(*Hr*$_o$) (δ) (*d*)	*Hr'* γ *c*	*Hr''* η *e*
R_z	*CDE*	+	+	+	(−)	−	−
R_1	*CDe*	+	+	−	(−)	−	+
R_2	*cDE*	+	−	+	(−)	+	−
R_o	*cDe*	+	−	−	(−)	+	+
(R_y)	(*CdE*)	(−)	(+)	(+)	(+)	(−)	(−)
R'	*Cde*	−	+	−	(+)	−	+
R''	*cdE*	−	−	+	(+)	+	−
r	*cde*	−	−	−	(+)	+	+

* *Wiener's nomenclature of antibodies (24).*

** *Fisher's nomenclature of antibodies (14).*

*** *Cappell's nomenclature of antibodies (2), which is used in this and succeeding papers.*

Fisher's hypothesis predicted two genes, R_y and R_z. R_z has since been isolated (12). It predicted two antibodies, anti-*d* and anti-*e*. The latter has since been discovered (11). The hypothesis is the most comprehensive offered, and has been useful in unifying the results of past researches, as a guide to further genetic study, and in predicting new findings. We have made use of it in the study of the *Rh* factor in Canadians of Japanese race reported here.*

Experimental

DETERMINATION OF GENE FREQUENCIES

Materials

Studies of the *Rh* blood type of persons of Japanese race have been made by Waller and Levine (19), and by Miller and Taguchi (10), each using three sera (anti-*D*, -*C*, and -*E*), and by Graydon, Simmons, Heydon, and Bearup (6) using anti-*D*. The data on the *Rh* reactions to the above three sera plus anti-*c* reported here were based on blood samples obtained in the summer of 1945 from 606 individuals living in Manitoba and Western Ontario.

In 525 persons, blood was drawn by skin puncture into 3.8% sodium citrate and tested on the day of collection. For the remaining 81, it was drawn from

* *In the table each gene is shown as being associated with three antigens. Fisher (13) made the alternative suggestion that three genes at closely linked loci were involved. More recent investigations (5) have made this the more likely explanation, but it is simpler, in a study such as this, to handle each group of closely linked genes as a unit and to name the group as though it were a single gene.*

Another antigen at the C-c locus, antigen C^w, and a corresponding antibody, anti-C^w, have been identified by Callender, Race, and Paykoç (1), but are omitted from this table, as the specificity of our sera in this respect had not been determined. We are indebted to Dr. R. R. Race for sending us, prior to publication in Nature, the manuscript by Race, Mourant, and Callender in which their most recent information on the antigens, C, C^w, and c and the corresponding antibodies is summarized.

the vein into sterile vacuum tubes, allowed to clot, and shipped by mail or express. In these samples, none of which were haemolysed or contaminated on arrival, the tests were carried out one to three days after the blood was drawn.

Most of the sera used were generously provided by Dr. Louis K. Diamond, but, in addition, a vial of high titre anti-*c* serum was kindly sent us by Dr. Peter Vogel. We are deeply indebted to both. Without their assistance this study would not have been possible. The anti-*D* and anti-*c* contained only these antibodies and were untreated save for dilution and for neutralization of anti-*A* and anti-*B* agglutinins with Witebsky's solution. The anti-*C* and anti-*E* were of the common types, which contain, in addition, anti-*D* and a blocking antibody (14, 22). They were prepared by neutralizing anti-*A* and anti-*B* with Witebsky, followed by suitable dilution.

Method

The reactions were determined by a method described by Chown and Lewis (4), which is a simplified form of an earlier described method (3).

Results

The individuals used in this study were about half of a group that had been brought east from three rural settlements in British Columbia in 1942. Japanese immigration to Canada began about 1896. It reached its peak in 1906 to 1908 and then steadily diminished to a trickle. Census figures are: 1901, 4738; 1911, 9067; 1921, 15,868; 1931, 23,342; 1941, 23,149. About 95% lived in British Columbia. We understand that much of the basic population of Japan, from which the immigrants were derived, has consisted for centuries of more or less isolated and self-perpetuating communities. It therefore seems probable that both the original immigrant population in British Columbia, and the group under study, would be somewhat heterogeneous with respect to the *Rh* genes.

It would be expected then that the population studied would be less favourable for accurate determination of gene frequencies than the white populations that have been studied in North America and Great Britain. Furthermore the inclusion of many entire families introduces further heterogeneity. We have, therefore, in Table II, summarized the reactions of the entire sample of 606 and of a subsample of 217 persons who, so far as we can determine, are unrelated. This latter sample is considered more suitable for calculating gene frequencies, although there is, in most respects, a close agreement between the entire sample and the subsample.

Table III shows the 12 phenotypes demonstrable with the four sera used, and the 36 genotypes possible with the eight genes of Table I. Since the phenotypes R_o, R_y, R', $R'r$, and $R'R''$ did not appear in the 606 individuals studied, the frequencies of the genes R_o, R', and R_y were probably either zero or very small indeed. For purposes of calculation they have been disregarded, and, consequently, the genotypes shown in parentheses are assumed

to be absent. The two individuals observed to be of phenotype R_z have been assumed to have the gene R_z, which has been isolated, rather than R_y, which has not. The six of phenotype R'' were members of one family.

TABLE II

NUMBER AND FREQUENCY OF *Rh* PHENOTYPES BASED ON REACTIONS WITH FOUR ANTI-SERA

Reaction with antisera				Phenotype	Entire sample		Sample of unrelated individuals	
D	*C*	*E*	*c*		Number	Frequency	Number	Frequency
+	+	+	+	R_1R_2	252	0.4158	95	0 4378
+	+	−	−	R_1	205	0.3383	73	0.3364
+	−	+	+	R_2	78	0.1287	27	0.1244
+	+	−	+	R_1r	62	0.1023	20	0.0922
−	−	+	+	R''	6	0.0099	1	0 0046
+	+	+	−	R_z	2	0 0033	1	0.0046
−	−	−	+	r	1	0.0017		
					606		217	

TABLE III

THEORETICAL PHENOTYPES AND THEIR COMPONENT GENOTYPES

Phenotype	Reactions to antisera				Genotypes
	D	*C*	*E*	*c*	
R_1R_2	+	+	+	+	$\begin{cases} R_1R_2,\ R_1R'',\ R_zR_2,\ R_zR'',\ R_zr \\ (R_2R',\ R_zR_o,\ R_2R_y,\ R_yR_o)^* \end{cases}$
R_1	+	+	−	−	$\begin{cases} R_1R_1 \\ (R_1R') \end{cases}$
R_2	+	−	+	+	$\begin{cases} R_2R_2,\ R_2R'',\ R_2r \\ (R_2R_o,\ R''R_o) \end{cases}$
R_1r	+	+	−	+	$\begin{cases} R_1r \\ (R_1R_o,\ R'R_o) \end{cases}$
R''	−	−	+	+	$R''R'',\ R''r$
R_z	+	+	+	−	$\begin{cases} R_zR_z,\ R_zR_1 \\ (R_zR',\ R_zR_y,\ R_1R_y) \end{cases}$
r	−	−	−	+	rr
R_o	+	−	−	+	$(R_oR_o,\ R_or)$
R_y	−	+	+	−	$(R_yR_y,\ R_yR')$
R'	−	+	−	−	$(R'R')$
$R'r$	−	+	−	+	$(R'r)$
$R'R''$	−	+	+	+	$(R'R'',\ R_yR'',\ R_yr)$

* *Genotypes shown in parentheses assumed to be absent from population under study.*

The calculations of gene frequencies based on the sample of 217 persons are:

$$R_1 = \sqrt{0.3364} = 0.5800 \text{ or } 58.00\%$$

$$R_1r = 0.0922$$

$$r = \frac{0.0922}{2 \times 0.5800} = 0.0795 \text{ or } 7.95\%$$

$$R''r = 0.0046$$

$$R'' = \frac{0.0046}{2 \times 0.0795} = 0.0289 \text{ or } 2.89\%$$

$$R_2R_1 = 0.0046$$

$$R_2 = \frac{0.0046}{2 \times 0.5800} = 0.0040 \text{ or } 0.40\% .$$

By difference $R_2 = 0.3076$ or 30.76%.

The genotypes of the individuals of phenotypes R'' and R_2 were known to be $R''r$ and R_2R_1 respectively through studies of their children as shown in the family studies.

Discussion of Results

Table IV shows the recalculation of the genotypes and phenotypes from the gene frequencies. The observed absolute frequency of phenotype R_1R_2 is greater, and that of R_2 less, than their calculated frequencies. (The small

TABLE IV

GENOTYPE AND PHENOTYPE FREQUENCIES IN SUBSAMPLE OF 217 INDIVIDUALS, CALCULATED FROM GENE FREQUENCIES

Genotype		Phenotype				
Name	Calculated frequency, %	Name	Frequency, %		Absolute frequency	
			Observed	Calculated	Observed	Calculated
R_1R_2	35 68					
R_1R''	3.35					
R_2R_2	0 25	R_1R_2	43 78	39.36	95	85.4
$R''R_2$	0.02					
R_2r	0 06					
R_1R_1	33.64	R_1	33.64	33 64	73	73.0
R_2R_2	9 46					
R_2R''	1.78	R_2	12.44	16 13	27	35.0
R_2r	4 89					
R_1r	9.22	R_1r	9.22	9.22	20	20.0
$R''R''$	0.08	R''	0.46	0.54	1	1.2
$R''r$	0.46					
R_2R_2	0.00	R_2	0.46	0.46	1	1 0
R_2R_1	0.46					
rr	0.63	r	0 00	0.63	0	1.4

discrepancy in the *r* phenotype is not surprising). Comparison of the observed and calculated absolute frequencies of phenotypes by the chi square test, with $n = 1$, gives a P value of .04, so that the deviations of observed from calculated results are statistically significant. Wiener, Unger, and Sonn (29) have shown that discrepancies of this kind (excess of R_1R_2 and deficiency of R_2 phenotypes) have been due, in some experiments, to the anti-*C* serum carrying traces of anti-*D* antibody.

It seems not unlikely that, in the present study, some of the blood samples of genotype R_2R_2, being homozygous for the antigen *D*, reacted to traces of anti-*D* in anti-*C* serum. These would then give the reactions of phenotype R_1R_2, with increase in the frequency of that phenotype and decrease in phenotype R_2.

Further evidence on this point can be had by examining the reactions of the 217 bloods to anti-*C* and anti-*c*, using the formulae employed in studies of *M* and *N* factors and substituting the terms *C* and *c* for *M* and *N*. (The formulae are summarized and explained by Graydon (31)). This allows all bloods to be divided into three categories in respect to Fisher's pair of allelic elementary antigens *C* and *c*, namely genotypes *CC*, *cc*, and *Cc*, which are comparable to the three genotypes *MM*, *NN*, and *MN*, respectively, and can be handled statistically in the same way.

Our data are: $CC = 74$; $Cc = 115$; $cc = 28$.

The calculated frequencies of the elementary antigens *C* and *c* and relative statistics are shown in Table V.

TABLE V

FREQUENCY OF ELEMENTARY ANTIGENS *C* AND *c*

Frequency of elementary antigens		D	σD	$D/\sigma D$	χ^2	P
C	*c*					
0.6060	0 3940	+0 0568	0 0339	1 676	2.615	0.11

The divergence of these results from those expected on the basis of the theory of inheritance is not very great, but in a population such as this, which is almost certainly heterogeneous for the factors under study, a negative value of *D* is expected. Therefore something has probably modified *D* in a plus direction to a greater extent than is at first suggested by Table V. False positives with the anti-*C* serum due to presence in it of anti-*D* antibody could bring about this result by increasing *Cc* at the expense of *cc*, so tending to make the value of *D* positive.

The probable extent of this increase may be indicated by calculating the expected percentage of positive reactions with anti-*C* serum from the observed negative reactions with anti-*c* serum. From Table II the latter is 0.3410.

Expected anti-C + = $1 - [1 - \sqrt{0.3410}]^2 = 0.8270$ or 82.7%, whereas, again from Table II, observed anti-C + is 87.1%.

In Table VI is set out a comparison of the phenotype and gene frequencies obtained in previous studies (10, 19), in which only three sera were used, with those found in the present study, in which four were used. In general

TABLE VI

COMPARISON BETWEEN PREVIOUS AND PRESENT STUDIES†

Investigators	Number tested	Phenotype frequencies, %							Gene frequencies, %							
		R_1 and R_1r*	R_2	R_1R_2	R''	R_z	$R'R''$**	r	R_1	R_2	R_o	R'	R''	R_y	R_z	r
Waller and Levine (19)***	150	37 4	13 3	47 3	0	0	0 7	1 3	61 8	37 8	0	0	0	0	0	0.4
Miller and Taguchi (10)***	180	51 7	8 3	39 4	0	0	0	0 6	70 2	27 7	0	0	0	0	0	2.1
Weighted average of above	330	45 2	10 6	43 0	0	0	0 3	0 9	66 4	32 3	0	0	0	0	0	1 3
Present study	217	42 8	12 4	43 8	0 5	0 5	0	0	58 0	30 7	0	0	2 9	0	0 4	8 0

† *Study of Graydon et al. (6) not included as only anti-D antiserum was used on all but two specimens.*

* *Since anti-c antiserum was not used in the previous studies, phenotype R_1 was not distinguished from phenotype R_1r. The two are therefore set out as a single group in this table.*

** *Using only three sera this phenotype includes genotypes R_yR_y, R_yR'', and R_yr as well as $R'R''$ and $R'R_y$. Gene R' or R_y or both must exist in this population, though in extremely low frequencies indeed.*

*** *As calculated by Wiener, Zepeda, Sonn, and Polivka (30).*

there is good agreement between our data and the weighted averages of the two previous studies. In ours two additional genes are indicated, R'' and R_z (Wiener, Zepeda, Sonn, and Polivka (30) had predicted the existence in Japanese of R_y or R_z or both). The greatest difference is in the calculated frequencies of the gene r. Our calculation is based on the frequency of the phenotype R_1r, which, as we did not find evidence for genes R' and R_o, we assumed to be genotype R_1r. This we think is a more precise method than derivation from the rare genotype rr. Wiener and his associates (30) also avoided the latter method, but were unable to make use of derivation from the phenotype R_1r since the data available to them did not distinguish between phenotypes R_1 and R_1r. They therefore derived the frequency of gene r by difference, and obtained much lower values than in our study. Had they used derivation from the weighted average frequency of genotype rr they would have obtained a frequency for gene r of 9.5, quite close to our 8.0%. The true frequency can only be determined by further studies.

Finally it is of interest to examine our data as they bear on Fisher's (13) hypothesis of three closely linked loci. The discovery by Callender, Race, and Paykoç (1) of a third allele, C^w, at Fisher's *C-c* locus (13) strongly supports this hypothesis.

Fisher and Race (5) have pointed out the possibility that the rare genes R_o, R', R'', and R_z in British populations are maintained by crossovers between the elementary antigens *c*, *d*, *e* in the common double heterozygotes R_1r, R_1R_2, and R_2r, and the gene R_y by similar crossovers in the triple heterozygotes R_1R'', R_2R', and rR_z. Their data on *Rh* gene frequencies are in remarkable agreement with this idea.

The hypothesis of crossovers can best be tested by family studies carried out on a far more extensive scale than heretofore employed, in order to detect rare crossovers in the generation in which they occur. Supplementary evidence will be obtained from populations, but it will be of a less certain nature.

The sample of 217 Japanese under study is admittedly much too small to make a satisfactory test. The genotypes R_1r, R_1R_2, and R_2r are the common double heterozygotes but have different frequencies from the British population. However, if the rates of crossing over are the same in both populations the frequency ratios R'/R_1r, R''/R_2r, and R_z/R_1R_2 will remain the same within limits of chance variation. Based on the frequency ratios obtained by Fisher and Race (5) in the British population, the expected frequencies of rare genes arising as crossovers in the Japanese population are as follows (our own data in parentheses):

R'	0.0022	(0 0000)
R_z	0.0042	(0.0040)
R''	0.0083	(0.0289)
R_o	0.0147	(0.0000)

The main discrepancy is the absence of R_o, but the values for R_z and R'' in our own data are derived from only one individual in each case. The above calculations are of interest only from the standpoint of method. We do not consider that they conflict with the hypothesis of crossing over.

In the Japanese population under study and in the population of Mexican Indians studied by Wiener, Zepeda, Sonn, and Polivka (30) the frequency of the double heterozygote R_1R_2 is higher than in the British population so that the hypothesis of crossing over predicts a higher frequency of the gene R_z. It is of interest that this condition is met in both cases.

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INHERITANCE OF THE ALLELOMORPHS OF THE *Rh* GENE IN CANADIANS OF JAPANESE RACE: A STUDY OF 65 FAMILIES[1]

By B. Chown,[2] Y. Okamura,[3] and R. F. Peterson[4]

Abstract

The blood of 129 parents and 182 children was tested by the four anti-*Rh* sera, anti-*D*, anti-*C*, anti-*E*, and anti-*c* (or anti-Rh_o, -*Rh'*, -*Rh''*, and -*Hr'*). The data obtained were in agreement with R. A. Fisher's hypothesis of the allelic *Rh* genes. No exceptions to expected results were found.

Introduction

In a previous paper (2) we reported the reactions of the blood of 606 Canadians of Japanese race to the *Rh* anti-sera, anti-*D*, anti-*C*, anti-*E*, and anti-*c* of Cappell (1), or anti-Rh_o, -*Rh'*, -*Rh''*, and -*Hr'* of Wiener (9), and, based on R. A. Fisher's (3) hypothesis of the allelomorphs of the *Rh* gene, derived the probable gene and genotype frequencies. In the present study we have used that portion of the same data that applied to families, i.e. 64 complete families, of 128 parents and 176 children, and one incomplete family, the mother and six children, to determine the inheritance of *Rh* genes.

The methods are detailed in the previous paper. In it we gave our reasons for believing that our anti-*C* serum contained a weak anti-*D* agglutinin, which, reacting with some homozygous R_2R_2 bloods, would result in some proportion of bloods that were actually of phenotype R_2 giving the reactions of, and being classed as, phenotype R_1R_2. While this must, if true, have some effect on the following data, this family study does not bring forth proof of the error.

In the same paper we also pointed out that the population dealt with was probably not homogeneous for the *Rh* genes. This does not affect the family study.

The same nomenclature for genes, genotypes, and phenotypes is used in the two papers.

Studies in the inheritance of the allelomorphs of the *Rh* gene in white families have been reported by Race and Taylor and their associates (4, 5), by Wiener (8), by Sonn and Wiener (6), and by Stratton (7). We have found no such studies on families of other races.

Results and Discussion

The results of our study are summarized in Table I, in which both matings and children are shown by phenotype. In our previous study it seemed

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TABLE I

SUMMARY OF INHERITANCE OF *Rh* PHENOTYPES

Type of mating (phenotypes)	No. of families	Phenotypes of children							
		R_1	R_2	R_1R_2	R_1r	R''	R_z	r	Totals
$R_1 \times R_1$	9	21							21
$R_1r \times R_1r$	2	1			1				2
$R_2 \times R_2$	2		2						2
$R_1 \times R_2$	3			3	3				6
$R_1 \times R_1R_2$	20	29		35					64
$R_2 \times R_1R_2$	4		4	3	1				8
$R_1R_2 \times R_1R_2$	14	9	6	22					37
$R_2 \times R_1r$	2		2	3					5
$R_1R_2 \times R_1r$	6	4	5	5	2				16
$R_z \times R_1$	1	2					1		3
?* $\times R''$	1					5		1	6
$R_1 \times R''$**	1			2					2

* *Father dead, Rh type unknown.*

** *This R'' is one of the five R'' offspring of the family listed above.*

probable that the genes R_o, R_y, and R' were absent from this population. In the following discussion of the matings, the same assumption is continued in order to eliminate repetition and undue complexity of statement. We realize that these genes may actually be present in exceedingly low frequencies.

$R_1 \times R_1$

The only possible genotype in the parents is, with the above proviso, R_1R_1. This then is the only possible genotype in the children. The 21 children were all of phenotype R_1.

$R_1r \times R_1r$

The results are those expected, as the two children provided two of the three theoretically possible genotypes. This mating and the one discussed above illustrate the value of using the four anti-sera, when possible, rather than the three (omitting anti-*c* or *Hr'*) as in Sonn and Wiener's (6) recent studies. Had only the three been used these two matings could not have been distinguished, so that it would have been necessary to make assumptions about the genotypes of parents and children.

$R_2 \times R_2$

The parental genotypes may be R_2R_2, R_2R'', or R_2r. The phenotype R_2 of the two children is the most probable according to theory.

$R_1 \times R_2$

The matings may be R_1R_1 by R_2R_2, R_2R'', or R_2r. The phenotypes of the two children (R_1R_2 and R_1r) are those expected.

$R_1 \times R_1R_2$

The R_1R_2 parents may have the genotypes R_1R_2, R_1R'', R_2R_s, $R''R_s$, or R_sr. The possible genotypes of the children are R_1R_1, R_1R_2, R_1R'', R_1R_s, and R_1r. As the gene R'' was found only in a single family to be described later, and as the expected incidence of R_1R_s and R_1r in children from this mating is extremely low, we may compare the observed phenotypes (29 R_1 and 35 R_1R_2) with the theoretical result of 32 R_1 and 32 R_1R_2. The chi square test gives a P value of approximately 0.5, indicating a satisfactory agreement.

$R_2 \times R_1R_2$

As expected, the phenotypes R_2 and R_1R_2 occurred in the children in approximately equal numbers. The one child of phenotype R_1r indicates that one of the R_2 parents was of genotype R_2r. Other genotypes are possible here but their expected incidence is extremely low.

$R_1R_2 \times R_1R_2$

Ignoring the theoretically possible but very rare phenotypes R'', R_s, R_1r, and r from this mating, the observed results (22 R_1R_2, 9 R_1, 6 R_2) may be compared with the theoretical values (18.5 R_1R_2, 9.25 R_1, 9.25 R_2). The chi square test, with $n = 2$, gives a P value of 0 4. The agreement is satisfactory.

$R_2 \times R_1r$

The approximately equal numbers of R_2 and R_1R_2 children are in accordance with expectation.

$R_1R_2 \times R_1r$

Ignoring rare genotypes, the four genotypes R_1R_1, R_2r, R_1R_2, and R_1r (corresponding to phenotypes R_1, R_2, R_1R_2, and R_1r) are expected in the ratio of 1 : 1 : 1 : 1. The observed numbers, 4, 5, 5, 2, when compared to this ratio by means of the chi square test with $n = 3$, gives a P value of 0.7, a good agreement.

$R_s \times R_1$

The more likely genotype of the R_s parent is R_1R_s since R_sR_s would be extremely rare. The phenotypes of the three children are those expected if the R_s parent is R_1R_s. (The same phenotypes would be expected if the R_s parent were R_1R_y).

$? \times R''$

It is apparent that the maternal genotype is $R''r$, and that the paternal genotype must have included the gene r. The most probable paternal genotype is $R''r$, giving the theoretical phenotypic ratio in the offspring of 3 R'' to 1 r.

$R_1 \times R''$

The phenotype observed is in accord with the theory and must be genotype

R_1R''

The data obtained in the family studies are in agreement with the theory of allelic *Rh* genes. No exceptions to expected results have been found.

Acknowledgment

We wish again to record our debt to Dr. L. K. Diamond and Dr. P. Vogel for supplying us with the sera. We are indebted to the Associate Committee on Medical Research of the National Research Council, Ottawa, for a grant covering the expenses of this investigation and for permission to publish it.

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THE BIOLOGICAL PURIFICATION OF VACCINE EMULSIONS BY PENICILLIN[1]

BY J. EDOUARD MORIN[2] AND HECTOR TURCOTTE[3]

Abstract

The present communication furnishes a new method for the purification of smallpox vaccine emulsion by penicillin. Penicillin, when added in definite quantities, possesses an undeniable antibiotic power on most of the Gram-positive cocci and on the vegetative forms of certain anaerobic bacteria such as *Clostridium perfringens* and *C. fallax* found in the vaccine. By maintaining a threshold of penicillin (56 units per cc.), the spores of the above mentioned anaerobic microbes cannot grow. When the vaccine emulsion freed from penicillin is inoculated, new strains without any pathogenic properties can be subcultured. Penicillin has no effect on the vaccine virus. By this technique, the vaccine can be freed, in a few days, of its principal pathogenic microbes except from Gram-negative bacilli. In the emulsion thus treated, the periodic control of the penicillin titre is as necessary as the repeated tests made to establish the potency of the vaccine.

Introduction

Of all the biological products used in the vaccination of human beings, smallpox vaccine requires the greatest care and attention in its preparation. The vaccine pulp produced by the calf *in vivo* is subject to all kinds of infection due to microbes from the surrounding area and the animal itself. In fact, even with the greatest care, the site of vaccination may become infected by microbes contained in the animal's excretions. Moreover, although the skin is thoroughly washed and carefully disinfected, there are always microbes of various kinds around the hair follicles and in the inner folds of the skin. Hence, it is necessary after collecting the vaccine pulp to sterilize this vaccine emulsion without killing the virus.

Up to now, it has been impossible to free these emulsions from the spore-bearing pathogens of gas gangrene and tetanus, and from hemolytic streptococci.

The Health Departments of all countries, by very precise laws, have banned the distribution of vaccine containing such bacteria.

Knowing the fundamental bacteriostatic powers of penicillin on most of the Gram-positive cocci and on certain gas gangrene microbes, we have tried

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to determine the action of penicillin as a purifying agent of glycerinated emulsions of vaccine virus containing a considerable amount of various microbes, including *Clostridium perfringens.*

Experimental Part

Purification of the Vaccine Pulp by the Addition of Penicillin

More than a year and a half ago work was undertaken on a vaccine that at first contained 150,000 bacteria per cc.: *Staphylococcus albus, Staphylococcus aureus, Streptococcus viridans, Streptococcus faecalis, Pneumococcus,* a Gram-negative bacterium and especially *Clostridium perfringens* and *Clostridium fallax* were found in the vaccine. The pathogenicity of each of these bacteria was determined by inoculation to guinea-pigs.

An injection of 0.1 cc. of *Clostridium perfringens* into the guinea-pig caused extensive necrotic lesions and several died within 28 hr.

In view of this important evidence and in conformity with the laws and regulations of the Department of Public Health at Ottawa, this vaccine had to be discarded.

(*A*) To this batch of vaccine (300 cc.) 130,000 units (433 units per cc.) of penicillin were added. The whole, thoroughly mixed for 10 min. in a Waring Blender was then placed for three days in a refrigerator at 23° F. (−5° C.). At the end of three days, 15 Smith fermentation tubes containing thioglycollate broth plus cooked meat were each inoculated with one cc. of the penicillin treated vaccine. After 16 days incubation at 37° C. all these tubes were found to be sterile.

(*B*) To determine the presence or absence of all pathogenic spores, the growth of which could have been prevented by penicillin, we inoculated to each of five guinea-pigs, 1 cc. of broth from these 15 tubes, which contained no trace of any microbe. Of these animals, only one died from pneumococcus pneumonia. There were, however, no lesions in the inoculated area. The four remaining guinea-pigs examined every two or three days showed no ill effects whatsoever, neither local nor general; under observation for two months, they continued to gain weight.

(*C*) Two months later, 25 tubes of culture were again inoculated each with one cc. of treated vaccine. On the seventh day of incubation at 37° C. five tubes contained gas.

Tube I contained a small Gram-negative bacterium.

Tube II contained a polymorphic bacterium with protoplasmic condensations; some of the organisms were slightly Gram-positive, others Gram-negative.

Tubes III and IV contained a large Gram-positive bacterium having all the morphological characteristics of the *Clostridium perfringens;* two guinea-pigs were inoculated with this strain, each receiving 1 cc. of the culture; no pathogenical effect was evident during the weeks that followed the inoculation,

but the animals died three months later during an epidemic, caused by *Proteus morganii*, that occurred in the animal room. It was also impossible to subculture, even on the most favourable aerobic and anaerobic culture media, these four gas-producing strains. Therefore these microbes can be considered as non-pathogenic.

Tube V contained a gas-producing strain of *Bacillus subtilis*.

One month later, 20 tubes were again inoculated with the treated vaccine; only two of these tubes became infected, one with a strain of *B. subtilis*, the other with a Gram-negative bacterium impossible to subculture.

Seven months later, using the same procedure, 10 fermentation tubes were inoculated with treated vaccine. Three days later, five contained a pure culture of *Clostridium perfringens;* however, a guinea-pig inoculated with 0.5 cc. of this strain presented no necrotic lesion.

The reappearance of this anaerobic microbe, although it is non-virulent, presented the following problems, which had to be immediately solved.

1. Was the quantity of penicillin added in the beginning sufficient?

2. Was the reappearance of *Clostridium perfringens* due to a progressive loss of the antibiotic power of the penicillin, inherent to the nature of penicillin itself, or due to the action of ingredients mixed in the pulp, such as glycerine and slight traces of phenol?

3. Or could spores persist under a certain titre of penicillin?

To solve those three problems, 100,000 units of penicillin were first added to the vaccine. A few days later, 10 tubes were inoculated with 1 cc. of the emulsion, and aerobic and anaerobic plates were made. One month later, another culture was effected under the same conditions. All tubes and plates remained sterile.

Titration of Penicillin in the Vaccine

To find an answer to the second problem, it was necessary, by periodic titrations to establish how great and how fast was the decrease of the antibiotic activity in the vaccine and also the factor responsible for this progressive loss.

Repeated titrations indicated without any doubt that 30 units of penicillin per cc. (15%) were inactivated in a week's time. The same results were obtained when titrations were effected on a simple glycerinated solution containing penicillin. The glycerinated solution mixed with the vaccine pulp is therefore one of the responsible factors for the diminution of the antibiotic activity of the penicillin. It is important to add a phosphate buffer to the solution.

Determination of the 'Threshold' of Penicillin

Moreover, the above experiments have allowed us to establish the important fact that vaccine pulp purified by penicillin should contain a minimal quantity of penicillin of 56 units per cc. Cultures remain sterile when inoculated with treated vaccine titrated with 56 units per cc. If 0.5 cc. of a very pathogenic

culture of *Clostridium perfringens* is added to the same pulp, all the cultures remain sterile.

On the other hand, if the number of units of penicillin is lower and the same quantity (0.5 cc.) of pathogenic culture of *C. perfringens* is added, after two days the growth of this organism is very abundant and large quantities of gas produced. However, the bacilli that have multiplied have lost all their pathogenic properties; in fact, guinea-pigs inoculated intramuscularly with 1 cc. showed no inflammatory reaction whatsoever.

Study of the Vaccine Emulsion Freed from Penicillin

In order to complete these experiments, we have endeavoured to ascertain the behaviour of the *Clostridium perfringens* in the vaccine emulsion from which penicillin had been completely removed. The vaccine was incubated at 37° C. for seven days, and then 15 tubes were inoculated always according to the same procedure (1 cc. of this product per tube). After 10 days' incubation, six tubes contained *Clostridium perfringens* and *C. fallax.* However, 0.1, 0.5, and 1 cc. of these strains inoculated to guinea-pigs proved to be non-virulent.

Inoculation of the Vaccine Emulsion Freed from Penicillin

Finally, we tried to determine whether the pulp freed of its penicillin after 21 days' incubation at 37° C. might contain bacteria or spores pathogenic for the guinea-pig. Sixteen guinea-pigs were each injected intramuscularly with 0.5 cc. of pure emulsion; these animals kept under close observation for several months presented no necrotic lesions.

These two experiments, the cultures and the inoculations of the pulp freed from penicillin, prove indisputably that the vegetative forms of bacteria, among which are *Clostridium perfringens* and *C. fallax,* are destroyed by penicillin, but that the pathogenic spores seem to persist and can grow again when the titre of penicillin drops below 56 units per cc. However, these new strains are not pathogenic. It is important that this be recognized. Thus it is possible to establish the following fact that *Bacteria especially Clostridium perfringens and C. fallax when impregnated with penicillin lose their pathogenic power.* This phenomenon is similar to the one observed by Pasteur when he incubated anthrax bacillus at 42.5° C.

Recovery of the Virulence of Clostridium perfringens

It is important to know whether these strains, having lost their pathogenic power under certain conditions can regain their virulence.

Experiments have shown that these new strains subcultured on blood agar and thioglycollate broth regain their former virulence after these transfers. In fact, guinea-pigs inoculated with 0.1 cc. died with typical necrotic lesions. This again can be compared to Pasteur's experiments with *Bacillus anthracis* when this microbe recovered its virulence after repeated passages in four- or five-day-old guinea-pigs.

One point demands further investigation. Do these new strains multiply by scissiparity or by sporulation? Results obtained on this question will be reported later.

Control of the Virulence of Vaccine Virus

The virulence of vaccine virus and its vaccination potency was continually tested throughout these experiments. It was necessary to ascertain whether penicillin could attenuate the virulence of this virus. The treated vaccine was periodically tested on the rabbit in successive dilutions up to 1/5000. At the same time, a control with a virus of known virulence was effected on the same animal. No differences were noted. Moreover, 35 children from 'La Crèche' were vaccinated with this treated vaccine at different intervals: all these children showed 'a perfect take'. Dr. de Varennes, who is in charge of this vaccination for the Health Department, has informed us that the children vaccinated with this treated vaccine showed much slighter inflammatory reactions than those that usually follow a vaccination with ordinary vaccine.

Conclusion

From all these experiments during which 110 cc. of vaccine virus were seeded on culture media and inoculated to 33 guinea-pigs, several important facts become evident.

1. Penicillin added to the vaccine emulsion in definite quantities possesses an undeniable antibiotic power on most of the Gram-positive cocci and on the vegetative forms of certain Gram-positive anaerobic bacteria, as *Clostridium perfringens* and *C. fallax*.

2. To prevent the growth of the spores of certain anaerobic microbes (*Clostridium perfringens* and *C. fallax*), it is necessary to maintain a definite threshold of penicillin (56 units per cc.).

3. Anaerobic bacteria treated with penicillin lose their pathogenic power.

4. Penicillin has no effect on vaccine virus.

5. All vaccinated children showed a much slighter reaction than when vaccinated with ordinary vaccine.

Advantages of This Method

(*A*) By this technique, vaccine virus emulsions containing hemolytic streptococci and anaerobic bacteria, which up to now had to be discarded, can be used.

(*B*) This vaccine can in a few days be freed of its principal microbes except from Gram-negative bacilli.

(*C*) By maintaining the required threshold of penicillin, all danger of infection at the time of vaccination may be avoided. The vaccines formerly used could always contain bacteria that the ordinary bacteriological methods did not reveal.

Periodic Control of the Titre of Penicillin

In using this technique, the titre of penicillin must be periodically verified as necessarily as the virulence of the vaccine virus is controlled.

Acknowledgment

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STUDIES OF SHOCK PRODUCED BY MUSCLE TRAUMA

II. PATHOLOGICAL CHANGES IN VARIOUS TISSUES[1]

By R. A. Cleghorn[2]

(With the Technical Assistance of Walter Cowan)

Abstract

The following pathological changes were found in the viscera of dogs that died as a result of muscle trauma: (*a*) *Lungs*—congestion and patchy consolidation that was not constant or extensive. (*b*) The mucosa of the upper *gastro-intestinal tract* and descending colon—congestion, haemorrhage, and at times small areas of erosion. (*c*) *Liver*—congestion of the sinusoids in dogs that died soon after trauma; in those dying later than three hours after trauma, degeneration of cord cells in the region of the central vein. Animals surviving longer than 18 hr. showed little or no cellular degeneration. (*d*) *Kidneys*—congestion of capillary tufts and other small vessels was seen in those dogs dying soonest. Sections from dogs that died three hours or more after trauma showed various degrees of degenerative changes in the tubules. (*e*) *Adrenal cortex*—congestion was apparent in animals dying within an hour of trauma. In later deaths, infiltration of leucocytes and degeneration of cells were notable features. Gross haemorrhages were present in dogs that died after five hours. Severe cortical changes were found in two dogs that bled to death accidentally at 8 and 10½ hr. after trauma despite a well maintained blood pressure up to the time of death; mild renal degenerative changes were present in both; a liver section obtained from one of these dogs appeared practically normal. (*f*) *Pancreas*—little congestion was seen in dogs that died within five hours after trauma but was more evident in dogs that died later and, in this respect, paralleled the gross changes in the adrenal glands.

The findings are discussed and it is concluded that absorption of toxic substances from the damaged muscles contributed, along with the secondary effects of oligaemia, to the tissue changes observed.

The object of this report is to describe pathological changes observed in the viscera of dogs that died following muscle trauma (6). These changes often were severe and developed in certain tissues earlier than in others. Some were seen in animals that died of a sudden haemorrhage some hours after trauma without an antecedent period of hypotension. Tissues from 40 animals were studied, the autopsy being done almost immediately after death. Blood cultures were obtained before or at the time of death in many cases. No difference was discernible in the pathological findings of animals showing positive cultures and those from which no growth was obtained.

Pathological Changes

1. *Local*

The skin at the site of injury was hyperaemic and moist. The legs became grossly swollen within an hour or two of trauma, but the swelling was still greater the next day in dogs that recovered. The muscle was not pulped,

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though some fibres were broken. Unclotted, serosanguinous fluid, and, occasionally, blood clots were seen when the muscle was cut.

2. *Remote*

(*a*) *Gross Changes*

The *heart* was stopped in diastole. Subpericardial ecchymoses were observed in one case, and may have escaped notice in others.

The *lungs* of some animals showed patchy, dark red, congested areas that usually were not extensive and were most evident in the lower lobes.

The *gastrointestinal tract* of animals dying within an hour of trauma was pale on the outside, resembling the appearance following acute haemorrhage, and the mucosa showed little or no congestion. In dogs dying one to five hours after trauma, mild to severe congestion of the mucosa was seen, bloody fluid was found in the lumen of the stomach and intestines, and small erosions of the mucosa were observed. The duodenum and descending colon were the most severely affected parts of the intestine. Massive congestion of the gastrointestinal tract was observed in dogs in which life had been prolonged by transfusion to three or four hours after trauma. Congestion and haemorrhage in the gastrointestinal tract usually were not so evident in untreated dogs that died later than five hours after trauma.

The *pancreas* was pale in animals that died within an hour or two after trauma. A slight to moderate degree of congestion was apparent in those living for a few hours. Marked congestion of this organ frequently was found in those dying later than 12 hr. after trauma.

The *liver* and *kidneys* of dogs dying a few hours after trauma were congested, particularly the cortical region of the kidney.

The *adrenals* of most dogs that died within an hour of trauma appeared normal. In those dying later, congestion was seen at the junction of the inner and middle thirds of the cortex; in some, there were streaks of haemorrhage radiating towards the capsule. Haemorrhage involving practically the whole cortex was seen in almost all animals that died later than five hours after trauma. In many of these there was equally gross haemorrhage in the medulla. Swelling of the gland and ecchymoses on the subserosal surface were seen frequently in later deaths. The right and left glands were affected similarly. The changes in the adrenal cortex were as pronounced in dogs in which life had been prolonged by a blood substitute adminstered three or more hours after trauma as in untreated dogs. When treatment was started early—one or two hours after trauma—and life was prolonged 10 hr. or more, the adrenals often did not show gross congestion or haemorrhage at death. Presumably early treatment prevented the development of such changes.

In Fig. 1 an attempt has been made to express graphically the degree of severity of the changes observed in the gross in different viscera in relation to the time after trauma when these changes were observed. The data were obtained from untreated dogs. The degree of intensity of congestion and

haemorrhage of each tissue was classified into three groups: 1, mild; 2, moderate; 3, severe; and the total for each group was averaged.

(*b*) *Microscopic Changes*

The *lungs*, *heart*, and *pancreas* showed intense packing of the capillaries with red blood cells. The *gastrointestinal tract* showed similar engorgement of mucosal capillaries but local haemorrhages and areas denuded of mucosa were seen also, particularly in the duodenum and colon.

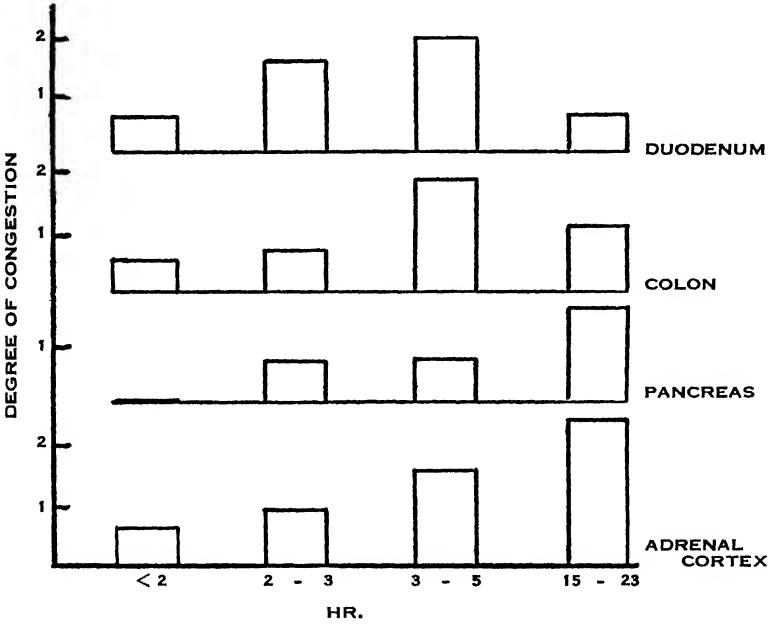


FIG. 1. *Order of development of congestion and haemorrhage in four tissues of traumatized dogs, as observed at postmortem examination. Each column represents average from eight dogs.*

In the *liver* there were various degrees of change. In dogs that died within an hour or so of trauma, the characteristic feature was an intense packing of red blood cells in the sinusoids. In some of these animals there were also signs of slight degeneration of cord cells about the central vein area. In dogs that died some hours later congestion was not so pronounced a feature but the degeneration about the central vein area was much more extensive (See Fig. 2). This was characterized by a breaking of cords, an increase in granularity, and pallor of cytoplasm, pycnosis, and swelling of nuclei. These changes apparently were dependent largely on the severity of impairment of the blood supply for, in one dog that accidentally bled to death from the carotid cannula 10½ hr. after trauma, the liver changes were much less evident than in many animals dying earlier (See Fig. 3). This dog had shown rather pronounced haemoconcentration and a heart rate of 200 for several hours, but the blood pressure had been 120 mm. Hg after trauma until a few minutes before death.

The most striking example of liver necrosis was seen in tissue from a dog that would have died about two and a half hours after trauma had a transfusion not prolonged life for four hours. The liver of dogs in which life was prolonged by transfusion beyond 20 to 24 hr. showed little or no degeneration in the central vein region, though congestion of the sinusoids—probably an agonal phenomenon—was observed occasionally (See Fig. 4). There was evidence of regeneration in the region of the central vein area in one animal killed on the third day when, apparently, it was recovering.

The *kidneys* of dogs that died in an hour or two after trauma showed congestion of the smaller vessels, including the capillary tufts. Vascular congestion seldom was present in dogs dying later. Evidence of tubular degeneration was observed in one dog that died three hours after trauma (Fig. 5) and in many others, both treated and untreated, that died at later periods. Frequent observations were hyaline and pigment casts in tubules and complete loss of epithelium throughout wide areas. The proximal tubules showed changes of varying severity from increased granularity and loss of cell outline to complete necrosis with casting off of a granular debris with nuclear remnants into the lumen. In some sections, as shown in Fig. 5, there were patchy areas throughout the cortex in which the tubules had lost all semblance of epithelium, the lumen being filled with homogenous pink-staining granular material. In this case the changes continued down to the distal and collecting tubules. Granular changes were not seen. Similar but less marked changes are shown in Fig. 6, which is of interest particularly as the section was obtained from a kidney of Dog 116, which showed marked liver necrosis (See Fig. 2). Renal changes also were observed in two dogs (Nos. 106 and 181) that bled to death accidentally at 8 and 10½ hr. after trauma, respectively. The blood pressure had been maintained above 110 mm. Hg in both dogs up to the time of death. The tubular degeneration was definite (Fig. 7) though it was not so marked as shown in Fig. 5. The kidneys of dogs transfused when hypotensive showed tubular degeneration irrespective of the prolongation of life effected, up to 24 hr. at least. The dog chosen as illustrative (Fig. 8) died 22 hr. after trauma. The renal changes were of the type described in Fig. 5. In this case they were less widespread and confined chiefly to the proximal tubules. The liver of this animal showed little deviation from normal (See Fig. 4).

The *adrenals* of an animal dying half an hour after trauma showed packing of red blood cells in the small vessels between the zona fasciculata and zona reticularis of the cortex. This was the mildest and earliest change observed. A more or less widespread invasion of the cortex by polymorphonuclear leucocytes was often seen in dogs dying a little later (See Fig. 9). Diffuse haemorrhages in the cortex were observed in most dogs dying three to five hours after trauma, and nearly always in those dying after five hours (See Fig. 11). Sometimes the haemorrhage was so extensive that the medulla was involved and the gland grossly swollen (See Fig. 12). The adrenal changes reached the maximum only in dogs that died 10 or more hours after trauma.

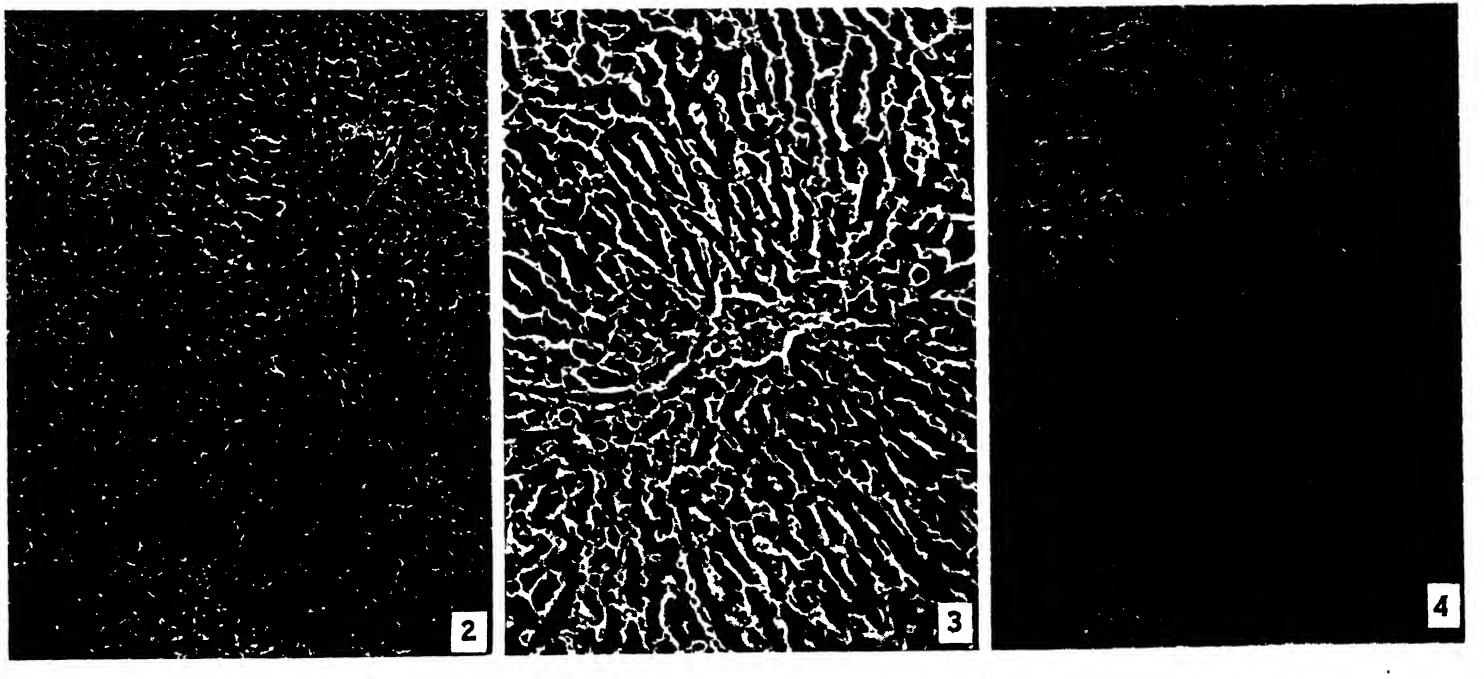


FIG. 2 *Section of liver (× 50) of Dog 116, which died 16 hr after trauma, without treatment Blood sugar at death 34 mgm %*

FIG 3 *Section of liver (× 110) of Dog 181, which died 10½ hr after trauma of accidental haemorrhage from the carotid cannula. Renal changes were slight (See Fig 7) Adrenal changes were very severe (See Fig 10)*

FIG. 4 *Section of liver (× 110) of Dog 233, which died 22 hr after trauma Life was prolonged by serum given three and one-half hours after trauma. Renal tubular changes were marked (See Fig 8) The adrenal cortex was haemorrhagic*

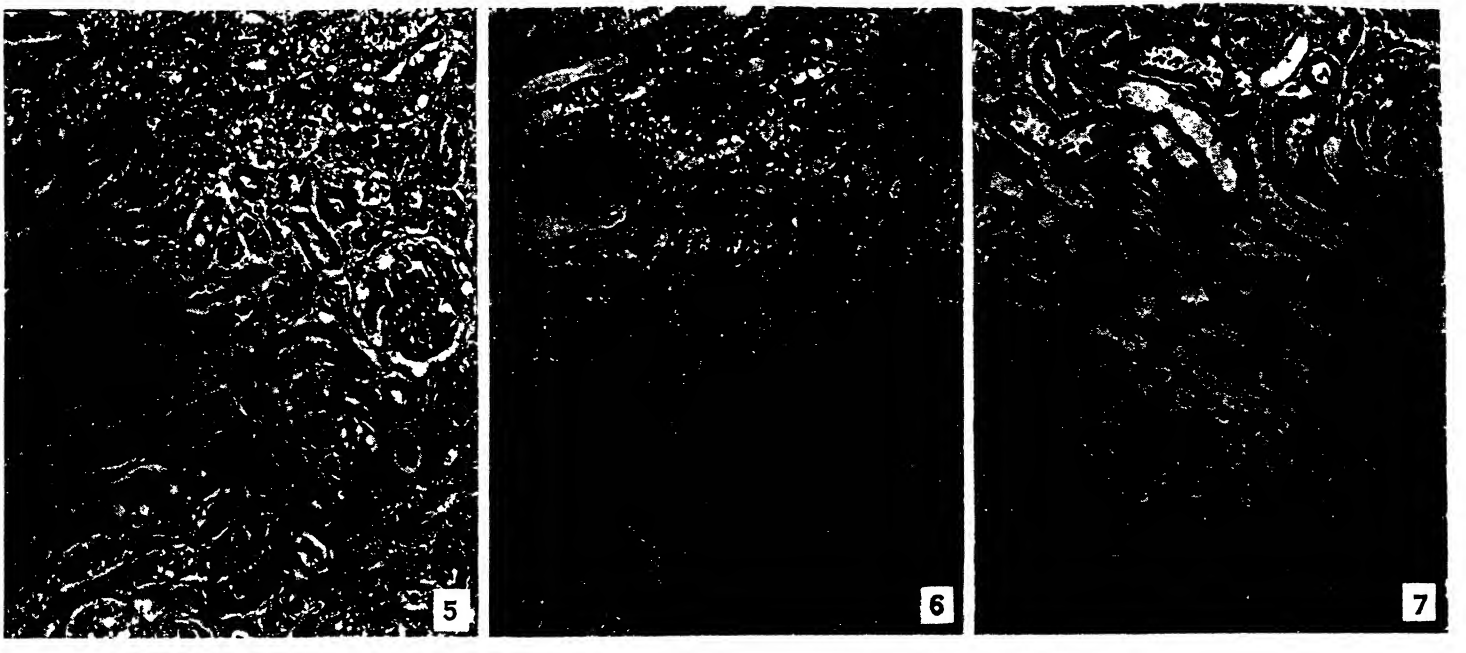


FIG 5 *Section of kidney (× 110) of Dog 74, which died three hours after trauma, untreated*

FIG 6 *Section of kidney (× 110) of Dog 116 which died 16 hr after trauma, untreated (See Fig 2, liver section of same dog)*

FIG 7 *Section of kidney (× 110) of Dog 181 which died 10½ hr after trauma, of accidental haemorrhage (See Fig 3, liver section, and Fig 10, adrenal cortex section, same dog)*

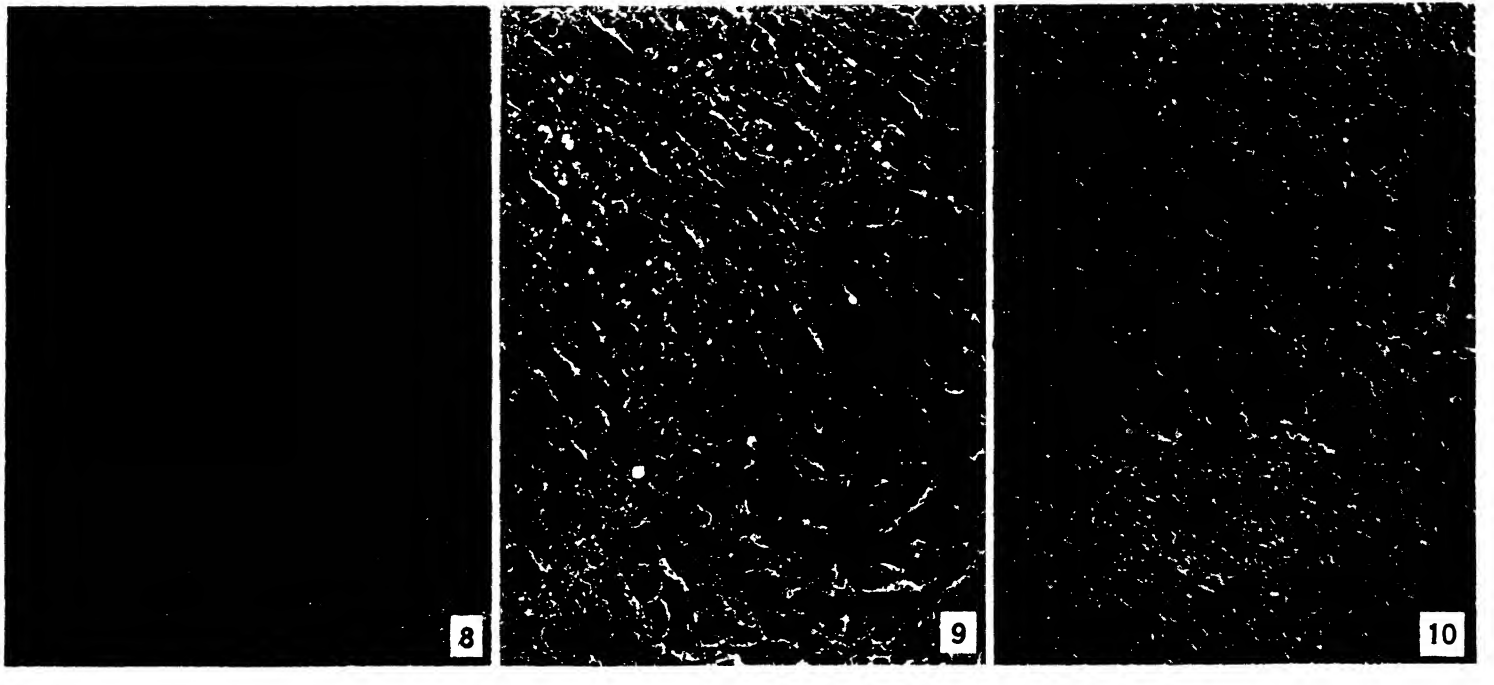


FIG 8 *Section of kidney (× 110) of Dog 233, which died 22 hr after trauma, life prolonged by serum given three and one-half hours after trauma (See Fig 4, illustration of liver that was practically normal)*

FIG 9 *Section of adrenal cortex (× 110) of Dog 3, which died two hours after trauma, untreated*

FIG 10 *Section of adrenal cortex (× 110) of Dog 181, which died $10\frac{1}{2}$ hr after trauma of accidental haemorrhage (See Fig 3, liver section, and Fig 7, kidney section, from same dog).*

Swelling and degeneration of the cells of the inner two zones of the gland and a heavy distribution of leucocytes were constant features in such cases. The adrenal cortical changes were not dependent upon a prolonged low blood pressure. This is shown by the findings in two dogs that died of accidental haemorrhage from the cannulated carotid artery at 8 and 10½ hr. after trauma: in both, the blood pressure had been maintained above 110 mm. Hg until death from haemorrhage took place. In the adrenal cortex of both these animals there were intense and widespread haemorrhages, cellular degeneration, and leucocytic infiltration of such severe degree that the architecture of the tissue was grossly altered (See Fig. 10). The gross appearance of the glands on section was like that of the gland in Fig. 11. In the kidneys of both dogs there was only slight congestion and a moderate degree of tubular degeneration (See Fig. 7). The microscopic appearance of the liver section obtained from one of these two dogs was practically normal (See Fig. 3). In both dogs the gastrointestinal tract was but mildly congested.

Discussion

It is known that prolonged anaesthesia with pentobarbital sodium contributes to tissue changes found in animals subject to trauma (2, 11). The more evanescent barbiturate pentothal sodium used in the present experiments can have played little part in the production of the pathological changes described above as the period of anaesthesia was brief and such extensive changes were not seen in dogs similarly anaesthetized prior to bleeding (5).

Moon (16) has implied that plasma loss occurs in various viscera in shock on the evidence of capillary engorgement and haemorrhage. Observations of Dunphy (11) and Cullen (7), and their associates, indicate that such changes do not precede but follow reduction in blood volume. The resultant physiological changes lead to anoxia, which may be the cause of capillary damage. These authors suggest that a toxic factor also may be operative in traumatic shock. This theory is supported by Freeman's observations that sympathectomy, which removes the vasoconstricting factor, prevents tissue changes in bled dogs (15) but does not have the same effect in those subject to muscle trauma (7, 14).

Fluid loss alone was insufficient to account for death in the muscle trauma experiments of Solandt and Best (19), in which measurements of increase in leg volume were made. The same technique for the production of trauma was used in our experiments but limb volume estimates were not done.

The intensiveness of the visceral changes found in the present experiments is believed to be due largely to the extensiveness of the muscle damage. Because the anaesthetic employed was evanescent, reflex vasoconstriction was relatively unimpeded compared to experiments conducted under prolonged narcosis. This means that the blood flow was probably more effectively reduced in sympathetically innervated areas following reduction in blood volume in our experiments. This may have contributed to the changes in such areas.

The degree of congestion of the lungs was not nearly so marked in the present experiments as in those of Fowler (13) in which shock was produced by intraperitoneally implanted muscle. Fowler, working in this laboratory, recognized and demonstrated that gross infection of the peritoneal cavity with *Clostridium welchii* occurred in dogs subject to the technique of Moon (16), who has not indicated whether infection was observed in his animals. It may be presumed that it was present and probably accounted for the great degree of congestion of the lungs reported in his as in Fowler's experiments.

The tissue changes observed in the present experiments did not develop simultaneously. Congestion of the gastrointestinal tract and of the adrenal cortex developed earliest. Changes in parenchyma of liver and kidney were seen next, though the liver often was relatively unaffected in longer surviving animals when kidney changes were well established. Thus, in cases in which the dogs lived a day or more, having been transfused before hypotension was of a serious order, renal changes were observed but the liver was essentially normal. Adrenal changes began earlier than in the liver but reached a maximum more slowly, paralleling the renal changes.

Oligaemia, due to local fluid loss at the site of trauma, and absorption of toxic substances from the traumatized muscle probably were the factors causing circulatory failure, which was the apparent cause of death in dogs dying a few hours after trauma. Potassium doubtless is one of the toxic substances released from traumatized muscle (4). The mechanism of other vasodepressor agents coming from crushed muscle has been discussed recently by Shorr (18). The occurrence of clostridial infection in the damaged muscle has been put forward by Aub (1) as an important cause of toxic absorption in this type of experiment.

In a previous paper (6) it was shown that some dogs died within 24 hr. of trauma with the development of low blood sugar. The hypoglycaemia can be correlated with the liver damage, which, as shown in the present paper, develops some hours after trauma, and probably is due, in large part, to anoxia.

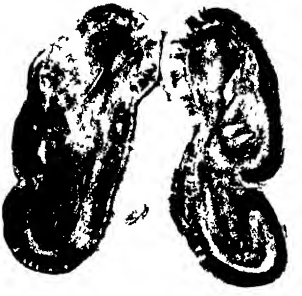
Disturbed renal and adrenal function, rather than a disturbance in liver function, probably accounted for deaths occurring after 24 hr., especially in transfused dogs, for their livers showed no such parenchymal damage. How closely the renal changes approximate those found in clinical cases of crush syndrome (3) or those described in dogs following tourniquet shock (10) cannot be estimated at the present time.

Severe adrenal changes were present in two animals in which there was no significant antecedent hypotension. Dunphy, Gibson, and Keeley (11) observed that microscopic changes in the adrenals of dogs developed before

FIG. 11. *Illustration of left adrenal of Dog 165, which died 22 hr. after trauma. This shows the degree of gross change most commonly seen in adrenals of dogs dying five hours and more after trauma.*

FIG. 12. *Illustration of left adrenal of Dog 151, which died 18 hr. after trauma, untreated. The gland is swollen and the medulla as well as the cortex involved by the diffuse haemorrhage.*

PLATE IV



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capillary engorgement of other abdominal viscera. Previously it had been shown (12) that such changes were not of neurogenic origin since they occurred in the transplanted gland. It seems that the adrenal cortex may be more susceptible to anoxia and to toxic substances absorbed from damaged tissue than many other organs, excepting the kidney.

No one, to our knowledge, has reported adrenals so extensively damaged in experimental shock as were constantly observed in our animals dying five and more hours after trauma. Other workers have observed and considered the adrenal changes in shock as highly significant. Donahue and Parkins (9) described depletion of lipoid and haemorrhage in the cortex following shock, and haemoconcentration induced by intraperitoneal glucose. They interpreted their findings as indicative of a marked reduction in functional efficiency of the cortex.

Pancreatic congestion paralleled the adrenal cortical changes in our animals; in some dogs that died after 24 hr., the pancreas appeared to be as congested as in dogs suffering from adrenal insufficiency. The reason for the association of pancreatic congestion and adrenal insufficiency has never been satisfactorily explained but it is a well established fact.

Attention should be drawn to the pathological findings in dogs subject to freezing of an extremity, reported by Muirhead and co-workers (17), and in animals suffering dehydration shock, as reported by Davis (8). In both instances the pathological changes resembled our findings in dogs following muscle trauma.

Acknowledgments

We are indebted to Prof. Duncan Graham for advice and criticism, to Prof. William Boyd for kindly arranging for the photomicrographs taken by Mrs. Vera Young of his department, and to Dr. John Hamilton for aid in interpretation of the sections. Figs. 11 and 12 are the work of Miss M. Wishart of the Faculty of Medicine Art Service, whom we wish to thank.

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SECTION E

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